BEHAVIORAL BUILDING BLOCKS: THE HORMONAL MECHANISMS BEHIND COOPERATION, PARENTAL CARE, AND PAIR BONDS IN BIRDS.

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Elizabeth McKenna Kelly

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BEHAVIORAL BUILDING BLOCKS: THE HORMONAL MECHANISMS BEHIND COOPERATION, PARENTAL CARE, AND PAIR BONDS IN BIRDS.

Elizabeth McKenna Kelly, Ph. D.

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Complex social behaviors have evolved multiple times independently across the vertebrates. An integrative approach to understanding how behavior evolves requires studying the underlying mechanisms. Across many species, cooperative breeding, parental care, and pair bonds share similar affiliative behaviors and likely share hormonal mechanisms but has yet to be determined in many species. I set out to study the proximate mechanisms of cooperative breeding, parental care, and pair bonding in three bird species, the cooperatively breeding Mexican jay (*Aphelocoma wollweberi*), the pair breeding Woodhouse's scrub jay (*A. woodhouseii*) and the pair breeding zebra finch (*Taeniopygia guttata)*. To determine if corticosterone concentrations correlate with cooperative breeding, I measured corticosterone in wild Mexican jays and Woodhouse's scrub jay across the breeding season. Corticosterone concentrations did not differ between Mexican jay helpers and breeders and were also not correlated with age and sex. Corticosterone did significantly increase with body mass in Mexican jays but there was no sex by body mass interaction. Corticosterone levels also did not differ between Mexican jays and Woodhouse's scrub jay. However, for both species, corticosterone significantly declined throughout the breeding season, with highest levels occurring in March and lowest levels during May and June. I also measured the effects of

experience and nonapeptides on both parental behaviors and pair maintenance behaviors by injecting a short-acting oxytocin receptor antagonist (OTA) or a saline control into breeding pairs of inexperienced or experienced zebra finches and experienced pair bonded zebra finches. For both the parental care and pair study, birds were injected over multiple days and then video taped to measure the effects of the injections on specific behaviors. For the parental care study, I also measured daily chick mass and chick mortality. I found that neither OTA nor parental experience affected time spent in the nest or nest maintenance. However, experience and OTA did affect time spent nest guarding, with inexperienced birds receiving the antagonist performing more nest guarding than inexperienced control and experienced antagonist birds. I also found that OTA significantly negatively affect chick growth rates and OTA and experience increased chick mortality, with experienced antagonist and inexperienced control birds having higher mortality than experienced control birds. For the pair maintenance study, I found the OTA reduced follow bouts in both sexes, allopreening in females, and song in males. The OTA did not affect clumping or pecking for either sex. Overall, this research sheds light on a the regulation and evolution of a number of complex social behaviors in birds.

BIOGRAPHICAL SKETCH

McKenna Kelly received her Bachelors in Science in May 2009 from the University of Illinois at Urbana-Champaign (UIUC), where she majored in Integrative Biology. While in undergrad, she spent a semester conducting research on savanna ecology in South Africa with the Organization for Tropical studies. She also researched marsupial limb evolution and development in the lab of Dr. Karen Sears and received High Distinction for her undergraduate thesis (Reduced integration in marsupial limbs and the implications for mammalian evolution). She continued to work with Dr. Sears during her Masters of Science at UIUC (2009-2011), successfully defending her thesis and publishing the paper “Limb specialization in living marsupial and eutherian mammals: constraints on mammalian limb evolution” in *Journal of Mammology*.

In the fall of 2011, McKenna joined the Department of Neurobiology and Behavior at Cornell University and switched taxa to work on the hormones of social behaviors in birds. Her research in the lab of Dr. Elizabeth Adkins-Regan has included studying the mechanisms behind cooperative breeding in two wild jay species, the role of sociality in numerical cognition in birds, and the role of nonapeptides in regulating pair bonds and parental care in zebra finches. Her work on jays led her to establish a field site and study flocks in the Chiricahua Mountains of Arizona.

This dissertation is dedicated to Brian Steidinger, who was with me every step of the way and helped me up when I stumbled. And to Malcolm Steidinger, who came into being in the middle of all of this and provided me with the motivation and perspective to finally finish (while also putting up a number of interesting road blocks).

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

Introduction

Complex social behaviors, including cooperative breeding, pair bonding, and parental care, have evolved many times across the animal kingdom (Clutton-Brock 1991, Cockburn 2006, Adkins-Regan and Tomaszycki 2007). In order to understand how these behaviors develop and evolve, we must understand the underlying mechanisms—the titular "building blocks"--that regulate each behavior. However, relatively little is known about the physiological basis for cooperative breeding, parental care, and pair bonding in many key taxa. Filling these gaps in our understanding requires novel descriptive studies and experimental manipulations.

More so than for pair bonding and cooperative breeding, the mechanisms underlying parental care have been studied in a number of species. Previous work on sheep (*Ovis aries*) and rats (*Rattus norvegicus*) establish an important role for nonapeptides (oxytocin and arginine vasopressin) in maternal care behaviors (rats-van Leengoed et al. 1987, Insel and Harbaugh 1989; sheep-Keverne et al. 1983, Da Costa et al. 1996). Similarly, in birds, mesotocin (avian homolog of oxytocin) regulates brooding in both turkeys (*Mealgris gallopavo*) (Thayananuphat et al. 2011) and chickens (*Gallus domesticus*) (Chokchaloemwong et al. 2013). However, these results do not predict maternal care across vertebrates, with mice showing no deficit in maternal care when oxytocin is disrupted (Young et al. 1996, Insel et al. 2001). Administration of mesotocin (amphibian homolog of oxytocin) in the biparental poison frog (*Ranitomeya imitator*) also had no effect on egg care behaviors (Schulte and Summers 2017).

In songbirds, mesotocin and arginine vasotocin (avian homolog of vasopressin)

do affect nest-building behaviors in zebra finches (*Taeniopygia guttata*), but in a sex specific manner. Female but not male zebra finches receiving an oxytocin receptor antagonist reduced nest-building behaviors, whereas a vasotocin receptor antagonist reduced nest building in both sexes (Klatt and Goodson 2013). Further complicating the role the nonapeptides play in regulating behavior, the measured effects of nonapeptide disruption can be dose dependent (Bales et al. 2007, Pedersen and Tomaszycki 2012). In birds, promiscuous binding of mesotocin (avian homolog of OT) and vasotocin (avian homolog of AVP) to multiple nonapeptide receptors also makes it difficult to distinguish between their effects on behavior. Birds have multiple vasotocin receptors and one OT-like VT3 receptor (Baeyens and Cornett 2006). In zebra finches, VT3 receptor will bind both mesotocin and vasotocin (Baeyens and Cornett 2006, Leung et al. 2009). Therefore, behavioral effects of targeting the VT3 receptor with an antagonist cannot isolate the activities of mesotocin, vasotocin, or both hormones in combination.

Birds are an excellent group for the study of the mechanisms of cooperative breeding, parental care, and pair bonding. Cooperative breeding is a common reproductive strategy in birds, found in approximately 9% of all bird species (Cockburn 2006), and has evolved multiple times independently (Koenig and Dickinson 2004). Birds also provide more parental care than any other vertebrate group, with 98% of bird species providing some type of parental care, including 80% providing biparental care (Cockburn 2006). The high percentage of birds that perform biparental care coincides with a high rate of social monogamy, with 95% of all bird species exhibiting social monogamy (Cockburn 2006). This correlation between social monogamy and parental care suggests that the two behaviors may be linked by the same physiological

mechanisms. I set out to determine the proximate mechanisms of cooperative breeding, parental care, and pair bonding in three bird species: the cooperatively breeding Mexican jay (*Aphelocoma wollweberi*), the pair breeding Woodhouse's scrub jay (*A. woodhouseii*) and the pair breeding zebra finch (*Taeniopygia guttata)*.

I examined the role the steroid corticosterone plays in reproductive suppression and cooperative breeding in Mexican jays and the role the nonapeptides play via the oxytocin receptor in regulating parental care and pair bonds in zebra finches. Corticosterone has been implicated in reproductive suppression in non-breeding helpers in a number of cooperatively breeding species (Saltzman et al. 1998, Young et al. 2006, Luo et al. 2015). Nonapeptides (oxytocin, vasopressin, and their non-mammalian homologs) are associated with a wide range of social behaviors across vertebrates (Donaldson and Young 2008, Choleris et al. 2013) including parental care (Bosch and Newmann 2012, Schulte and Summers 2017) and pair bonding (Insel and Hulihan 1995, Liu and Wang 2003). However, how (and whether) corticosterone regulates cooperative breeding and nonapeptides regulate parental care and pair bonding behaviors varies widely among vertebrates (corticosterone-Schoech et al. 1997, Young et al. 2006; mesotocin-Goodson and Thompson 2010, Smith et al. 2010).

The body of my dissertation is divided into three data chapters (plus one supplemental chapter that is included in the appendix) followed by a conclusion that synthesizes the results of these studies. Starting with Chapter two, I present the results of a study to determine whether wild Mexican jay helpers were reproductively inhibited due to glucocorticoid-mediated (corticosterone) stress imposed by Mexican jay breeders. I also tested whether corticosterone concentrations differed between closely

related but socially different species, the cooperative Mexican jay and the pair breeding Woodhouse's scrub jay. In Chapters three and four, I present the results of experiments examining how nonapeptides (mesotocin and vasotocin, avian homologue of oxytocin and vasopressin) regulate parental care (Chapter three) and long-term pair bonds (Chapter four) in zebra finches. In Chapter three, I demonstrate that both parental experience and nonapeptides affect parental outcomes. In Chapter four, I show how blocking nonapeptides affects certain long-term pair maintenance behaviors in experienced zebra finch pairs. I end with Chapter five, a summary of how these hormones regulate social behaviors in birds. I also include an appendix chapter on how sociality can affect cognition, specifically quantity discrimination, by comparing highly social Mexican jays with pair territorial Woodhouse's scrub jays.

Description of the Methods

I studied how hormonal mechanisms affect cooperative breeding, parental care, and pair bonding in birds. To understand the role corticosterone (a steroid hormone) may play in regulating helping behavior in cooperatively breeding birds, I measured and compared plasma corticosterone concentrations in Mexican jay breeders and helpers and pair breeding Woodhouse's scrub jays across multiple breeding seasons. I also analyzed the role sex differences, body mass, and seasonality may play in corticosterone concentrations of both species. To understand the role that nonapeptides may play in both pair bonding and parental care, I administered a nonapeptide receptor antagonist to experienced pair bonded zebra finches and both inexperienced and experienced zebra finch parents. I then measured whether birds receiving the antagonist changed their social behaviors: for parents, nest guarding, time spent in

nest, nest maintenance; for pair partners, singing, clumping, allopreening, and pecking. Additionally, I measured whether the antagonist negatively affected parental success by monitoring chick growth rate and chick mortality. Together these experiments tested how multiple proximate (hormonal) and behavioral factors affected complex social behaviors and reproductive outcomes in birds.

Study species

To determine the proximate mechanisms of cooperative breeding, parental care, and pair bonding, I studied three bird species: the cooperatively breeding Mexican jay (*A. wollweberi*), the pair breeding Woodhouse's scrub jay (*A. woodhouseii*), and the pair breeding zebra finch (*T. guttata*). The *Aphelocoma* genus is an excellent model group for exploring the hormonal basis of cooperative breeding. The social variation among *Aphelocoma* species spans obligate cooperative breeders to territorial breeding pairs and has been well studied in terms of the ecological factors that promote cooperative breeding (Brown 1963, Woolfenden 1975, Carmen 1989, Webber and Brown 1994). Both are omnivorous, caching species (McCormack and Brown 2008). However, the similarities between Mexican jays and Woodhouse's scrub jays belie a qualitative difference in their life history strategies. Mexican jays are plural cooperative breeders, where multiple females nest within a flock with several birds helping at each nest. These helpers are usually immature offspring of the dominant pair from the previous 1–2 years, but also include apparently unrelated flock members (Brown 1963, McCormack and Brown 2008). In contrast, Woodhouse's scrub jays are territorial, monogamous pair breeders that defend their territory from conspecifics during the breeding season (Curry et al. 2002). Mexican jays and Woodhouse's scrub jays have overlapping ranges at my

field site in SE Arizona (Peterson 1993, Hopp et al 2001), which allows me to control for climatic variability when comparing hormones between the two species.

Due to their extensive pair bonding and parental investment, as well as their readiness to pair and raise chicks in cages, zebra finches are an excellent species to explore the hormonal basis of pair bonding and parental care. Zebra finches form socially monogamous pair bonds and are highly motivated to pair and breed, even in lab settings. These pair bonds are long-term and will last until the death of one of the pair members; divorce is very rare in zebra finches (Adkins-Regan & Tomaszycki, 2007). Zebra finches form and maintain their pair bonds by physical contact (clumping), mutual grooming (allopreening), singing, and time spent brooding in the nest together. Both males and females participate in nest building, incubation, brooding, and feeding of chicks (Zann 1996).

During my dissertation research, I used an integrative, comparative approach to understand how multiple factors affected complex social behavior in Mexican jays, Woodhouse's scrub jay, and zebra finches. By studying how hormonal mechanisms, ecological factors, and individual experience affect specific social behaviors, my work has expanded our understanding of the how and why of social behavior.

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CHAPTER TWO

Seasonality, breeding status, and corticosterone concentrations in Mexican jays and Woodhouse's scrub jays

Abstract: Cooperative breeding, where helper individuals forgo breeding to assist in the feeding and care of the offspring of one or more breeding pairs within a group, occurs widely across the animal kingdom. According to the reproduction inhibition hypothesis, breeding behaviors in helper individuals are physiologically suppressed by elevated glucocorticoid levels. I tested whether wild Mexican jay (*Aphelocoma wollweberie*) helpers were reproductively inhibited due to glucocorticoid-mediated stress imposed by breeders. I also tested whether cooperatively breeding Mexican jays had similar corticosterone concentrations as a closely related sympatric species without helpers, Woodhouse's scrub jay (*A. woodhouseii*). The results do not support the reproductive inhibition hypothesis. Mexican jay corticosterone concentrations did not differ in helpers vs. breeders, males vs. females, or older vs. younger birds. Corticosterone concentrations also did not differ between Mexican jays and Woodhouse's scrub jays. However, for both species, corticosterone significantly declined throughout the breeding season, with highest concentrations occurring in March and lowest concentrations during May and June. Corticosterone concentrations were positively associated with body mass in Mexican jays, but not Woodhouse's scrub jays. These results suggest that Mexican jay helpers are not reproductively inhibited by corticosterone, but experience similar seasonal declines as Mexican jay breeders and non-cooperative Woodhouse's scrub jays.

CHAPTER 2: Corticosterone and Breeding Status in *Aphelocoma* Jays

Introduction

Cooperative breeding, where three or more individuals provide care for offspring, occurs in many taxa across the animal kingdom, including arthropods (Brockmann 1997), mammals (Lukas and Clutton-Brock 2012), fishes (Wong and Balshine 2011) and birds (Cockburn 2006). Why some individuals forgo direct fitness benefits to help breeders is the major research theme of the field. Whereas evolutionary theory has described numerous conditions under which cooperation should evolve (e.g., Axelrod and Hamilton 1981), these theories have treated the mechanisms that underlie behavior as a black box. Studying the mechanisms of a behavior do not merely illuminate the contents of the black box, but can alter the qualitative predictions of evolutionary models when the two are integrated together (van den Berg and Weissing 2015). Corticosterone (Cort) has been proposed as a potential hormonal mechanism of reproductive suppression in helpers, as adrenal glucocorticoids (GC; including Cort) secreted in response to chronic stress can also prevent reproduction (Moore and Miller 1984, Luo et al. 2015). Here I tested whether differences in Cort plasma concentrations correlated with differences in breeding status in cooperatively breeding Mexican jays (*Aphelocoma wollweberi*). Additionally, to determine whether differences in Cort are associated with differences in sociality, I also compared Cort concentrations between the social Mexican jay and the pair breeding Woodhouse's scrub jay (*A. woodhouseii*).

Parental care is costly (Clutton-Brock 1991, Santos and Nakagawa 2012) and breeders benefit when helpers forgo breeding to lighten the breeder's parental load (Heinsohn 2004). Breeders, therefore, have a strong incentive to recruit helper individuals and prevent them from breeding. Breeders can suppress reproduction in

helpers by denying them access to breeding opportunities (Nelson-Flower et al. 2013), increasing aggression towards helpers during breeding periods (Young et al. 2006), or inducing chronic stress in helpers to activate GC suppression (Luo et al. 2015). Helper reproduction inhibition due to chronically high GC varies widely across species; with some species exhibiting GC induced reproduction suppression (Saltzman et al. 1998, Young et al. 2006) whereas others find no evidence of GC suppression (Mays et al. 1991, Schoech et al. 1997). Thus, one prediction from the reproduction inhibition hypothesis is that helper Mexican jays should have elevated GC relative to breeding Mexican jays.

Alternatively, chronic stress due to fighting to maintain dominance position can lead breeders to exhibit significantly higher concentrations of GC than helpers (see Creel 2001). For breeders in these species, maintaining breeding physiology and social dominance are energetically demanding, leading to high chronic concentrations of GC (Romero 2002, Reeder and Kramer 2005b, Sands and Creel 2004). Therefore, though social dominance can lead to high reproductive output, this dominance comes at the cost of chronically high GC concentrations, which can be caused by dominance-associated aggressive interactions (Creel 2001, Sands and Creel 2004).

Additionally, differences in GC levels between breeders and helpers can be sex-specific. A recent meta-analysis of physiological mechanisms of alloparental care in cooperatively breeding carnivores found a sex-specific influence of dominance on GC levels, with breeding males having higher GC levels than male helpers, but with no clear pattern for GC levels in females across carnivora (Montgomery et al. 2018). This pattern was reversed in cooperatively breeding Florida scrub jays (*A. coerelescens*), a closely

related species to Mexican jays and Woodhouse's scrub jays, as female breeders exhibited significantly higher concentrations of Cort than non-breeding females, but with was no difference in GC levels between male breeders and helpers (Schoech et al. 1991). Thus, as an alternative to the reproductive inhibition hypothesis, it is possible that Mexican jay breeders will have elevated GC relative to Mexican jay helpers although this may be sex specific.

Further complicating matters, GC concentrations can differ due to age, sex, seasonality, and ecology (Wingfield 2006, Reeder and Kramer 2005b). Recent work in passerines found that seasonal changes in concentrations of Cort (from non-breeding to breeding season) were positively associated with body mass across a number of species (Casagrande et al. 2018). Therefore, as Mexican jays are larger than Woodhouse's scrub jays (Carmen 1989, Brown and Brown 1990), Mexican jays may experience a larger change in Cort concentrations from non-breeding to breeding season compared to Woodhouse's scrub jays. Additionally, during the breeding season, smaller bodied species tend to have higher concentrations of Cort than larger bodied species (Bokony et al. 2009, Hau et al. 2010). Cort concentrations may therefore be higher in Woodhouse's scrub jays compared with Mexican jays during the breeding season.

I measured whether individuals with different breeding behaviors differed in GC concentrations by measuring circulating Cort concentrations in cooperatively breeding Mexican jays (*A. wollweberi*) and closely related pair breeding Woodhouse's scrub jays (*A. woodhouseii*). By comparing between breeders and helpers within a species (Mexican jays), I tested whether Cort was elevated or suppressed in helper vs. breeding

birds. Additionally, by comparing Cort concentrations at different times of the year among species (Mexican jay vs. Woodhouse's scrub jay), spanning before and during the time of breeding, I tested whether seasonal differences in breeding-related demands were associated with Cort concentrations in breeding and non-breeding birds.

Methods

Study species

I measured Cort concentrations in two bird species, the cooperatively breeding, group living Mexican jay (*A. wollweberi*) and the territorial, pair breeding Woodhouse's scrub jay (*A. woodhouseii*). Mexican jay helpers are typically offspring from previous clutches, though unrelated flock members also help (Brown and Brown 1990). All individuals for both species were wild and free throughout this study. At the time each bird was caught to take a blood sample, none of the birds had been involved in any experiments.

Study site

This study was conducted in the foothills of the Chiricahua Mountains near the Southwestern Research Station in Portal, AZ (31° 53'N, 109° 12'W). The Mexican jays inhabit woodland dominated by oak-juniper-pine while the Woodhouse's scrub jays live in mesquite dominated scrub. During the study (3/4/13-4/3/15), samples were taken across the months of March-June. The majority of Mexican jays were sampled during March (27 total) and April (18 total), with fewer birds sampled during May (5 total) and June (7 total). Similar sampling patterns occurred for Woodhouse's scrub jay with more birds sampled during March (5 total), April (8 total), and May (5 total) and fewer

sampled in June (1 total) across the experiment.

Study design

Birds were caught using baited Potter's box traps, bled immediately after capture, measured, and banded with a unique band combination. I took samples from 57 individual Mexican jays from six flocks (7-14 members per flock) and 19 Woodhouse's scrub jays. After banding, I determined breeding status through individual observations and nest observations. I assigned breeding status for Mexican jays and Woodhouse's scrub jays based on behavioral observations of nest-building (performed solely by the breeding pair) (Barken et al. 1986, Brown and Li 1995), egg laying, diurnal incubation (performed mainly by the breeding female, Brown and Brown 1990), and feeding of the nestlings and fledglings (performed by the breeding pair and helpers of both sexes, Brown and Brown 1990). For Mexican jays, if an individual was involved only in feeding nestlings/fledglings but not in nest-building/egg-laying/incubation, I assigned them "helper status". I assigned "Unknown" when I could not accurately determine breeding status. It is likely that many "Unknown" Woodhouse's scrub jays were breeders as most Woodhouse's scrub jays were found regularly in pairs on territories though I had great difficulty finding their nests.

Sex was determined for both species based on genetic data extracted from DNA by the Cornell Lab of Ornithology following the protocols established for the Florida scrub jay (Fridolfsson and Ellegren 1999, Schoech et al. 2007, Rensel and Schoech 2011). Briefly, a polymerase chain reaction (PCR) was used to amplify a conserved intron of the chromo-helicase-DNA binding protein (CHD1) gene, present in most birds as CHD1W on the W chromosome and CHD1Z on the Z chromosome. The intron differs

in size between the two gene copies. PCR primers 2550F and 2718R amplify both gene copies but produce an approximately 500 base pair product from the CHD1W gene and an approximately 700 base pair product from the CHD1Z chromosome. Thus, PCR from genomic DNA produces two bands from females and one from males when visualized on an agarose gel, allowing sex determination based on these PCR products. Overall, there were 25 female and 32 male Mexican jays and 4 female and 15 male Woodhouse's scrub jays.

In Mexican jays, birds of different ages have different bill coloration, with adolescents under the age of 2 years having pink/white streaks in their bills while adults over the age of 2 years have solid slate colored bills (Brown and Brown 1990, Brown et al. 1997). I assigned birds to either adolescent (under 2 years) or adult (over two years) age category based on their bill coloration at the time of sample collection.

In total, the Mexican jay sample size included: 12 female breeders, 11 male breeders, 8 female helpers, 16 male helpers and 5 females and 5 males with unknown breeding status male with unknown breeding status. For Woodhouse's scrub jay: 1 female breeder, 5 male breeders and 3 females and 10 males unknown for breeding status. Unknown Mexican jays and Woodhouse's scrub jays were excluded from analyses where breeding status was required (comparing helpers to breeders) but were included when breeding status was not being compared (age comparisons for Mexican jays and species by date and body mass comparisons for both).

Blood Collection and Extraction

Once trapped, I quickly collected blood samples to reduce the likelihood of elevated Cort due to handling stress. Average time from capture to blood collection for

Mexican jays was 214.88 seconds (± 54.89 seconds STD) and 232.39 seconds (70.07± seconds STD) for Woodhouse's scrub jays. I used heparinized micro-hematocrit tubes to collect blood samples after puncturing the wing vein with a 26-gauge needle. After collecting blood, I measured head length and breadth, overall head-bill length, nares to bill tip, culmen, bill breadth and width at nares, wing-chord, tail length, and body mass. I also counted head parasites, took a fat score, and looked for brood patches and cloacal swellings. All samples were collected between 0700 and 1200 hours MST. After collection, blood samples were placed in a cooler and kept chilled with icepacks until transport to the laboratory (usually within 1-2 h) where the plasma was separated by centrifugation. Plasma was stored at -20C until shipped on dry ice to Cornell University for analysis.

Radioimmunoassay

Cort was measured using the MP Biomedicals Double Antibody Corticosterone Radioimmunoassay (07-120103). Assays were run by the Diagnostic Endocrinology Lab of Cornell University. Each plasma sample was diluted 1:50 (3 ul plasma + 147 ul diluent), then run in duplicate. All samples for both species were run in a single assay. For the intra-assay CV, two different pooled jay samples were run, five times in duplicate. The mean concentrations for the samples were 15.24 and 88.68 ng/ml and the intra-assay CVs were 10.1 and 3.9%, respectively. Parallelism was demonstrated with the standard curve by serially diluting jay plasma with the assay buffer. The assay sensitivity was determined to be 0.61 ng/ml.

Statistical analyses

Prior to analysis, one outlying Cort value (>3 SD from the mean) was excluded (Rensel and Schoech 2011). Cort concentrations were not normally distributed for either species (Fig. 1A). Therefore, I log-transformed Cort concentrations for all subsequent analyses (Schoech et al. 2007, Rensel and Schoech 2011) (Fig. 1B). Cort concentrations increase rapidly in birds due to handling stress (Schoech et al. 1991, Romero and Romero 2002, Schoech et al. 2007). For both species, I measured the increase in log-transformed plasma Cort concentrations with time from capture to completion of bleeding (hereafter "handling time", *sensu* Neter et al. 1985, Schoech et al. 1991). To measure the effects of handling time, I ran a linear model with log transformed Cort as the response variable and handling time (in seconds) as the predictor. I also tested whether changes in Cort with handling time were significantly different in both species by including species and species by handling time interaction effects. To correct for the effects of handling time in all subsequent analyses, I included handling time as a covariate in analyses of covariance (Small et al. 2017). For graphical purposes, I account for the effect of handling time by plotting the residual log-transformed plasma Cort concentrations (actual - expected log transformed Cort from Figure 2).

I ran separate ANCOVAs with log-transformed plasma Cort concentrations as the response variable and different fixed-effect terms in linear models, depending on the hypothesis being tested (with handling time as the covariate in each case) (Table 1). I compared Cort concentrations within Mexican jays in four separate models, one each that included as a main effect (1) breeding status (breeder vs. helper) by sex, (2) age (birds < and > 2 years old) by sex, (3) sex (males vs. females), and (4) sex by body

mass, with birds of unknown status (10 unknown breeding status) omitted from the breeding status comparison. For comparisons between species, I ran three separate ANCOVA models with log-transformed Cort as the response and as main effects either (1) species or (2) species x day of year (such that January 1 is day 1), and (3) species x body mass (in g). All data analyses were performed using R studio (Version 1.0.136).

In order to generate standardized effect sizes for all main and interaction effects, η^2 ("eta squared") values were calculated using the "lsr" package in R. The values of η^2 give the proportion of the total variance explained by each model term. Additionally, in order to assess the magnitude and uncertainty around the differences in group means (e.g., between breeding, sex, and age groups in Mexican jays), Hedge's *g* statistic was calculated along with 95% confidence intervals using the "effsize" package in R (Hedges and Olkin 1985). Hedge's *g* gives the difference of group means divided by the pooled standard deviation (*g*=1 means that the means differ by 1 standard deviation); in general, Hedge's *g* does not differ significantly from Cohen's *d*, except for at sample sizes <20. Following Cohen (1988), when $g<0.50$ the effect size is considered small; though this convention has to be interpreted with caution, as small standardized effect sizes can still be biologically important. In general, when the 95% confidence intervals around Hedge's *g* do not intersect with zero, group means are significantly different at $p<0.05$. As Hedge's *g* can only be used for between group comparisons of means, for comparisons of slopes between species for how Cort concentrations change with the day of year, non-standardized effect sizes for the difference in slopes (in units of residual log[Cort]/day) were calculated with 95% confidence intervals using the "lsmeans" package in R.

Results

Increase in Cort with handling time

Cort concentrations increased with handling time across all samples (η^2=0.155, F_{73}=9.463, P=0.002) (Fig. 2). Neither species nor species by handling time had a significant effect on Cort concentrations *(P*>0.05), indicating that both species have equal slopes describing how Cort concentrations increase with handling time.

Comparisons within Mexican jays: Breeding Status, Age, and Sex

Mexican jay plasma Cort concentrations did not significantly differ between breeders and helpers (η^2=0.053, F_{41}=2.54, P=0.118) and there was no significant sex by breeding status interaction effect (η^2=0.01, F_{41}=0.48, P=0.492) (Fig. 3A). Cort concentrations also did not significantly differ between young and old birds (less than and greater than 2 years old, respectively) (η^2=0.052, F_{51}=3.175, P=0.08) and there was no significant sex by age interaction effect (η^2=0.011, F_{51}=0.678, P=0.413) (Fig. 3B). Females and males also did not significantly differ from one another (η^2=0.007, F_{53}=0.444, P=0.507) (Fig. 4A). There was a significant increase in Cort concentrations with body mass (η^2=0.0005, F_{51}=4.22, P=0.045), though the sex by body mass interaction effect was not significant (η^2=0.012, F_{51}=0.785, P=0.379; Fig. 4B).

Comparisons between Mexican jays and Woodhouse's scrub jays: overall differences, changes with seasonality, and changes with body mass

There was no overall species difference in plasma Cort concentrations between Mexican jays and Woodhouse's scrub jays (η^2=0.017, F_{44}=0.04, P=0.842) (Fig. 5A). Over the course of the breeding season (March-June), plasma Cort concentrations

significantly decreased for both species (η^2=0.076, F_{70}=10.979, P=0.001) (Fig. 5B). The species by day of year interaction effect was not significant (η^2=0.011, F_{70}=1.003, P=0.32) (Fig. 5B). When modeling both species, there was no overall effect of body mass on Cort concentrations for either species (η^2=0.03, F_{70}=2.633, P=0.109) (Fig. 6). The species by body mass interaction effect was also not significant (η^2=0.03, F_{70}=2.628, P=0.109) (Fig. 6).

Confidence Intervals:

For comparisons of Cort concentrations between Mexican Jays and Woodhouse's Scrub Jay and among groups within Mexican Jays (breeders vs helpers, males vs females, <2 yr vs >2 yr olds) that were non-significant, effect sizes were small (Hedge's g<0.40, Table 2), with 95% confidence intervals intersecting with zero (consistent with the absence of significance of these model terms as determined by ANOVA, Table 1). For the comparison between Woodhouse's Scrub Jay and Mexican Jays of how Cort concentrations significantly change with day of year (seasonality), the difference in slopes was estimated as -0.376 (95% confidence interval, CI, range: -0.743 to -0.009 in units residual log[Cort]/day).

Discussion

I tested whether plasma Cort concentrations were elevated or depressed in helpers relative to breeders in cooperatively breeding Mexican jays. In contrast with the predictions of the glucocorticoid (GC) reproduction inhibition hypothesis, Mexican jay breeder and helper Cort concentrations did not significantly differ from each other, with reproductive stage explaining ~ 5% of the total variability in Cort concentrations, with a

Hedge's *g* of <0.40 (indicating small effects) for the difference in mean Cort between breeders and helpers. This negative result with respect the GC reproductive inhibition hypothesis of helper Mexican Jays is consistent with previous work in closely related Florida scrub jays (*A. coerelescens*) (Schoech et al. 1991, Schoech et al. 1997) and other cooperatively breeding birds (white-browed sparrow weavers*, Plocepasser mahali,* Wingfield et al. 1991*;* Harris's hawks*, Parabuteo unicinctus,* Mays et al. 1991). Instead, though this study did not measure how Cort levels affected helping behaviors, the lack of difference in Cort concentrations between breeder and helper Mexican Jays is consistent with the Parent-Helper Hypothesis of alloparental care, which predicts that adolescent helpers undergo the same endocrine changes as parents to facilitate helping (Schradin et al. 2018).

The lack of difference in Cort concentrations I observed between Mexican jay helpers and breeders at different ages and sexes may be due to the relatively stable flock relationships within cooperatively breeding *Aphelocoma* jays (Brown and Brown 1990). Stable territory and breeder-helper relationships are found in the closely related Florida scrub jays (Woolfenden and Fitzpatrick 1977), which also do not exhibit differences in Cort concentrations between male breeders and helpers (Schoech et al. 1991, Schoech et al. 1997). Group instability and dominance reorganization lead to increases in GC levels in many group living species (Sapolsky 2005). Therefore, the lack of difference between Cort concentrations in Mexican jay helpers and breeders may be a consequence of group stability buffering birds from breeding-status associated stress.

Plasma Cort concentrations can change with life stage (Wingfield 2006) and social dominance position (Creel 2001). In Mexican jays, age and sex influence dominance position, with adolescents and males holding higher dominance positions than older and female birds, respectively (Barkan et al. 1986, Brown et al. 1997). Because stress caused by dominance-associated aggressive interactions can increase plasma Cort (Creel 2001), I also compared Cort concentrations in Mexican jay males vs. females and younger vs older birds. I found no statistically significant effect sof age or sex on plasma Cort in Mexican jays (which explained ~5 and <2% of the variance in Cort, respectively). Once again, these negative results mirror a similar study in Florida scrub jays, which also found no difference in Cort concentrations when comparing birds of different ages (Wilcoxen et al. 2011).

Cort concentrations in Mexican jays do increase with body mass, although there was no sex by body mass interaction effect and there was no relationship between body mass and Cort for Woodhouse's scrub jay. This result differs from previous work in Florida scrub jays, where heavier birds had lower concentrations of Cort than lighter birds (Schoech et al. 2007). Though I did not measure social dominance, the observed increase in Cort concentrations as body mass increased may be driven by an individual's position within the flock. In Mexican jays, males tend to be dominant to females (Barkan et al. 1986) and males tend to be slightly larger than females (Pitelka 1951). A recent meta-analysis of the hormonal mechanisms of cooperatively breeding carnivores found that dominant males tend to have higher glucocorticoid levels than subordinates (Montgomery et al. 2018). Though I did not find a sex by body mass

interaction effect, the increasing Cort concentrations with body mass may be driven by larger birds holding higher positions within the flock.

I also compared plasma Cort concentrations between Mexican jays and Woodhouse's scrub jays across the breeding season. Although plasma Cort concentrations do not significantly differ between the two species when taking the day of year into account, Cort in both species significantly declines from early March (during nest building and egg laying) into late June (following the hatching of chicks and fledging). The observed seasonal declines in Cort are consistent with previous studies in free-living birds (Astheimer et al. 1995, Romero et al. 2006, Lattin et al. 2012), although notably not in Florida scrub jays (*A. coerelescens*) (Schoech et al. 1991, Schoech et al. 1997). Mexican jays and Woodhouse's scrub jays may experience seasonal changes in Cort due to living in the desert, a more harsh and unpredictable environment, than the scrub/suburban environments of Florida scrub jays. Indeed, many of the initial studies on seasonal changes in Cort focused on birds living in arctic or desert environments (Romero 2002; but see Lormee et al. 2003, Wada and Shimizu 2004, and Romero et al. 2006 for some more recent examples for seasonal changes in Cort in less extreme environments).

Life history stage and a species' current vs. future reproductive investment can also influence seasonal changes in Cort. The GC seasonal plasticity hypothesis proposes that seasonal changes in GC concentrations are related to a species' investment in current vs. future reproduction (Casagrande et al. 2018). For species that invest relatively more in current reproduction, GC concentrations are higher during the breeding season (egg-laying through fledging) than the non-breeding season (anything

after offspring independence through mating, including nest building) (Bokony et al. 2009, Hau et al. 2010). In birds, the greatest increase in GC concentrations during the breeding season occurred in species investing in large clutches (used as a proxy for reproductive investment) (Casagrande et al. 2018). As both Mexican jays and Woodhouse's scrub jays have relatively small clutches (1-6 eggs) (Carmen 1989, Brown and Brown 1990), their lack of increase in GC concentrations during the breeding season is consistent with the GC seasonal plasticity hypothesis. For Mexican jays, breeders may also invest less in current reproduction due to assistance from helpers. Indeed, superb fairy-wren (*Malurus cyaneus*) females breeding in the presence of helpers lay smaller eggs and produce smaller chicks than females in the absence of helpers (Russell et al. 2007). Therefore, in cooperatively breeding species, breeders may reduce investment in current offspring to favor future offspring, without paying a cost in reduced offspring viability.

The higher concentrations of plasma Cort found in Mexican jays and Woodhouse's scrub jays at the end of the non-breeding season/beginning of the breeding season may be due increased energy demand. During this time, birds must repeatedly defend their territories from intrusions by other pairs and flocks while also establishing nests and laying eggs. As the glucocorticoids (including Cort) play an important role in energy mobilization (see Landys et al. 2006), the higher Cort concentrations in both species may allow them to respond more quickly to territorial intrusions and prepare to lay eggs. In great tits (*Parus major*), males and females with high Cort concentrations during egg-laying and low Cort concentrations during the nestling stage had higher reproductive output than birds that did not reduce their Cort

concentrations during the breeding season (Ouyang et al. 2013). In some species, GC concentrations are suppressed during the breeding season to reduce the likelihood of nest abandonment (Romero 2002). As elevated Cort concentrations can reduce or disrupt parental care (Wingfield and Kitaysky 2002, Love et al. 2004, Angelier et al. 2009), Mexican jays and Woodhouse's scrub jays may need to reduce their individual Cort concentrations after egg laying in order to care for chicks.

Conclusions

Overall, I determined that Cort concentrations do not differ among Mexican jays of different breeding status, age, and sex. I found no support for the glucocorticoid reproduction inhibition hypothesis or the social dominance glucocorticoid hypothesis. Cort concentrations in Mexican jays do increase with body mass, although there was no sex by body mass interaction effect. Both Mexican jays and Woodhouse's scrub jays experienced a significant decline in Cort during the breeding season and the two species did not differ in Cort concentrations. These results support recent work in other passerines, where helpers and breeders in cooperatively breeding birds do not differ in Cort concentrations and individuals experience a decline in Cort during the breeding season.

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Figures

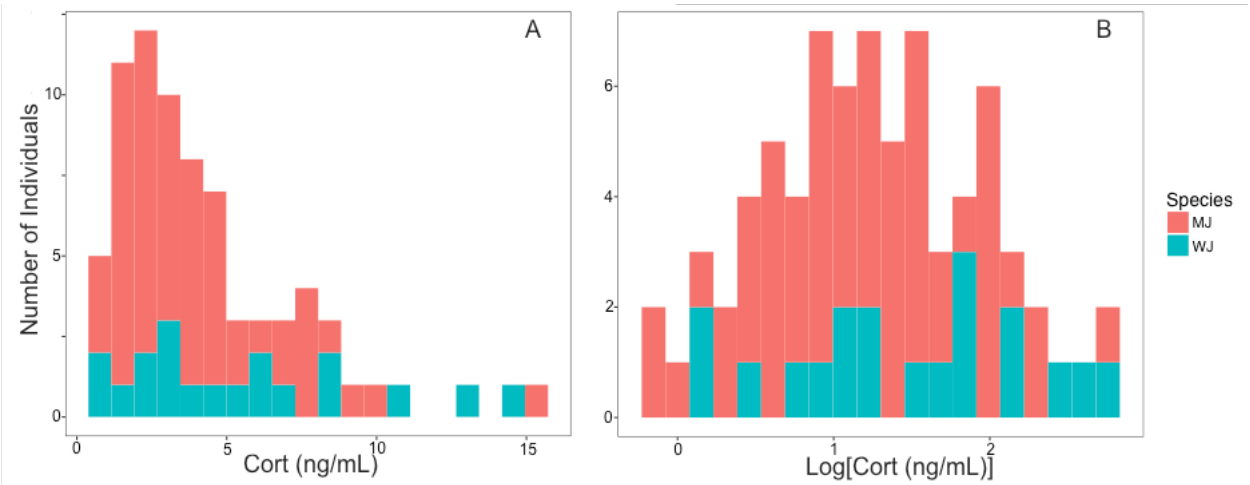

Figure 1AB. Raw plasma Cort concentrations (A) and log-transformed plasma Cort concentrations (B) for all individuals. Mexican jays (MJ) are pink and Woodhouse's scrub jays (WJ) are blue.

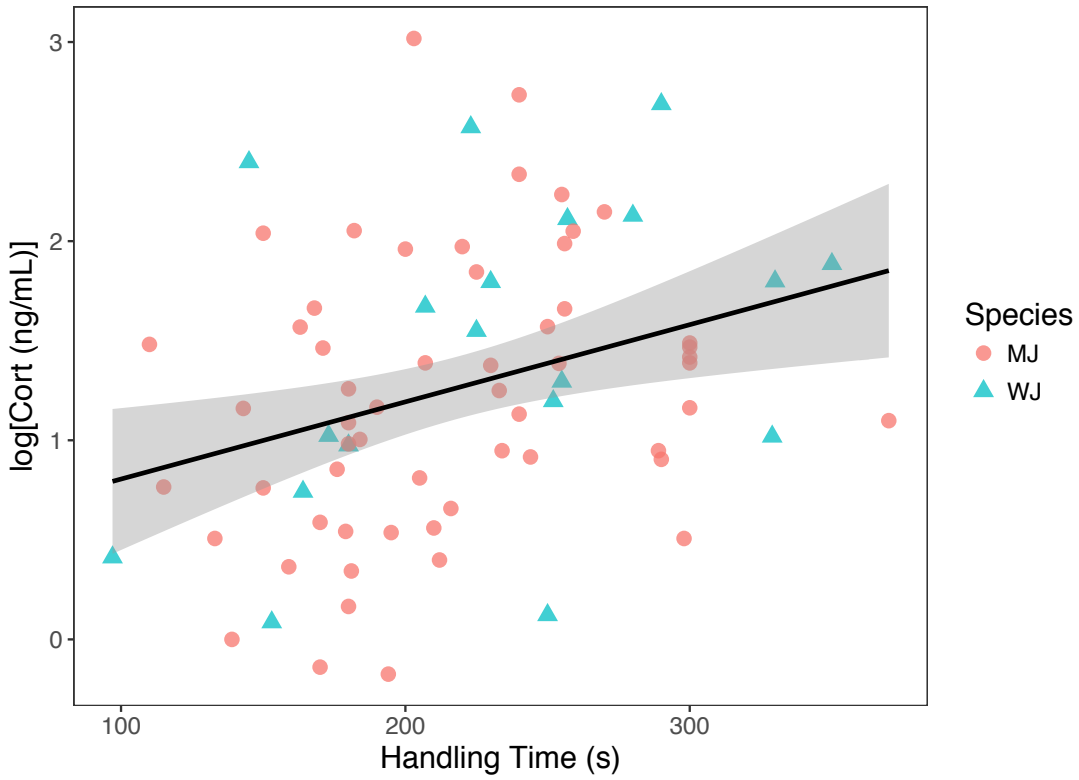

Figure 2. The effect of capture and handling time on plasma Cort concentrations in Mexican jays (MJ, pink) and Woodhouse's scrub jays (WJ, blue). The grey shaded region represents +/- one standard error. As handling time increased, plasma Cort concentrations increased (*P*=0.002).

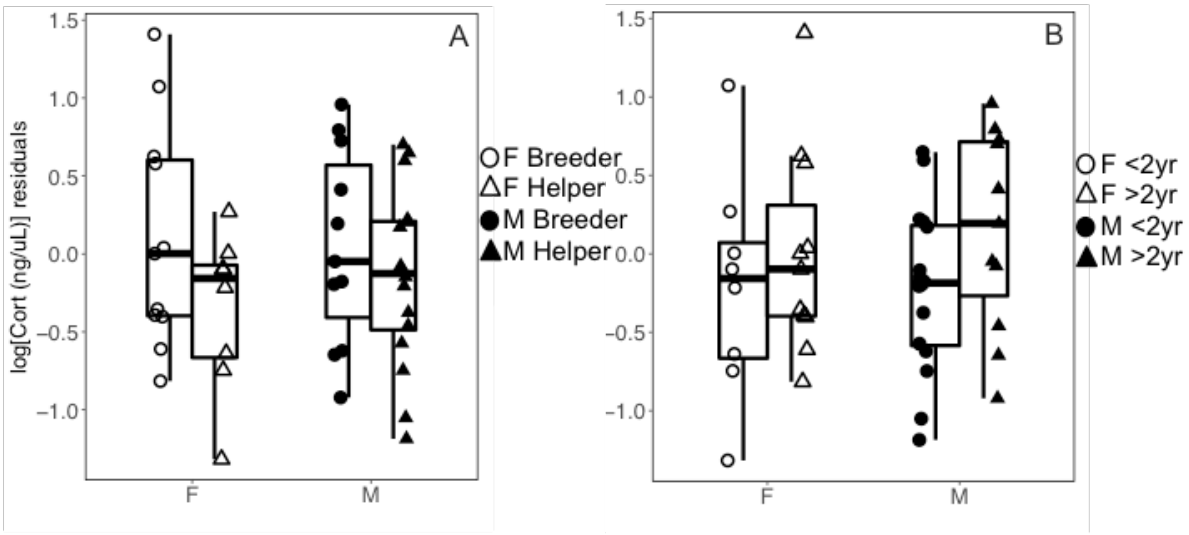

Figure 3AB. Comparison of residual plasma concentrations of Cort after accounting for handling time between Mexican jay breeders and helpers by sex (A) and adolescent Mexican jays (<2yr old) and adult Mexican jays (>2yr old) by sex (B). Boxes represent the first, second and third quartiles while the lines expand 1.5 times the inter-quartile range. Plasma Cort concentrations did not significantly differ between helpers and breeders (P=0.118) and there was no breeding status by sex interaction (P=0.492). Cort concentrations also did not differ between adolescent and adult Mexican jays (P=0.08) and there was no age by sex interaction (P=0.413).

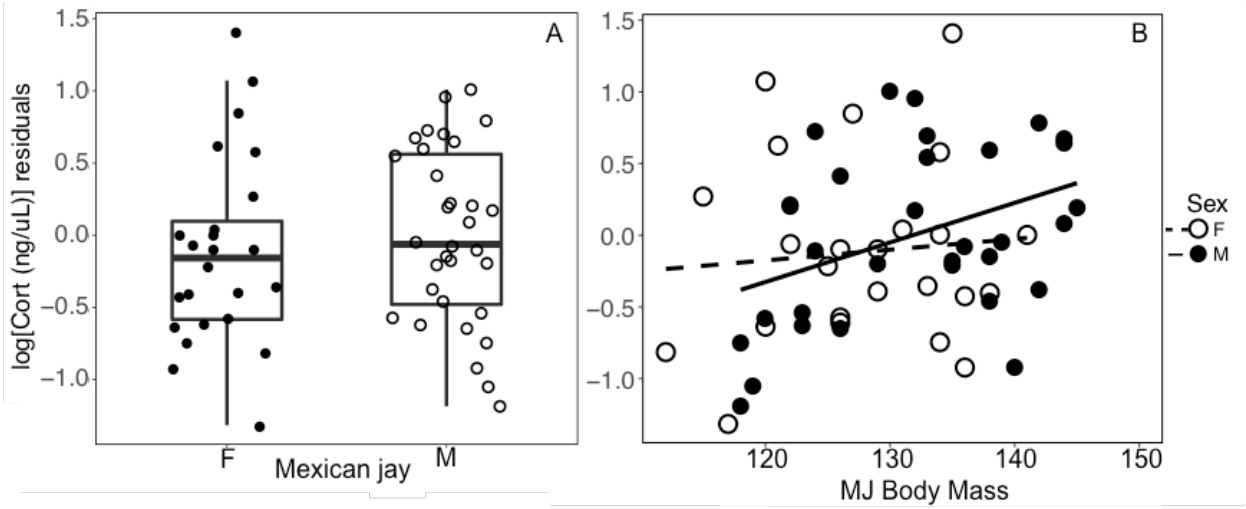

Figure 4AB. Comparison of residual plasma Cort concentrations between Mexican jay females and males (A) and sex by body mass interaction effect (B). Female and males did not differ significantly from each other (*P*=0.507) (A). Mexican jay Cort concentrations did significantly increase with body mass (*P*=0.045) but the sex by body mass interaction effect was not significant (*P*=0.379) (B).

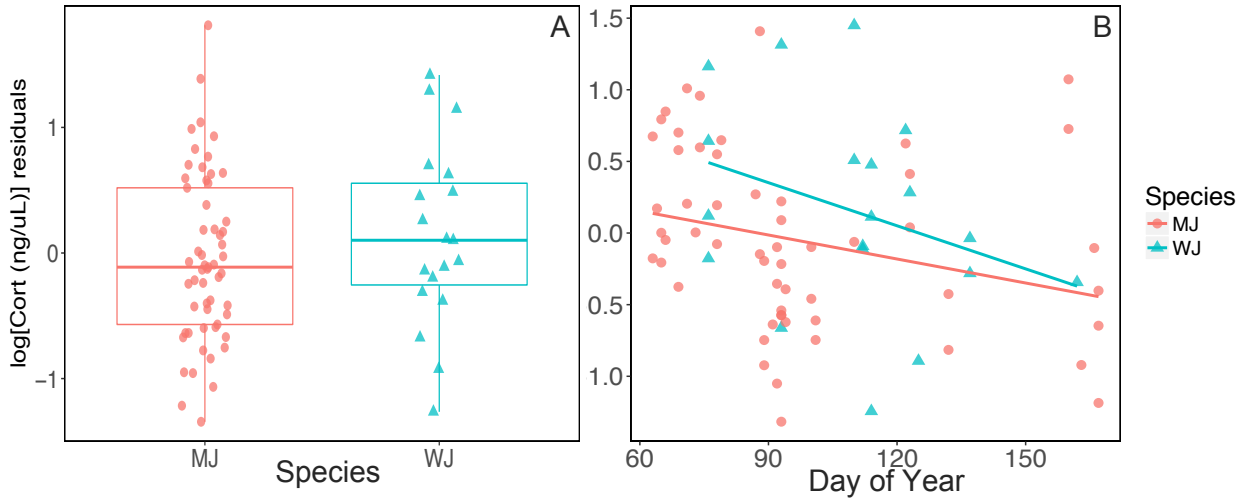

Figure 5AB. Comparison of overall residual plasma concentrations of Cort between Mexican jays (MJ, pink) and Woodhouse's scrub jays (WJ, blue) (A) and across season for both species (B). Plasma Cort concentrations did not significantly differ between the two species (*P*=0.107). For panel B, numbers on the X-axis refer to days across the breeding season, with January 1st set as day 1 (March 1st is day 60). Cort concentrations significantly declined for both species across the breeding season (*P*=0.001). There was no significant difference when comparing species by seasonality (*P*=0.32).

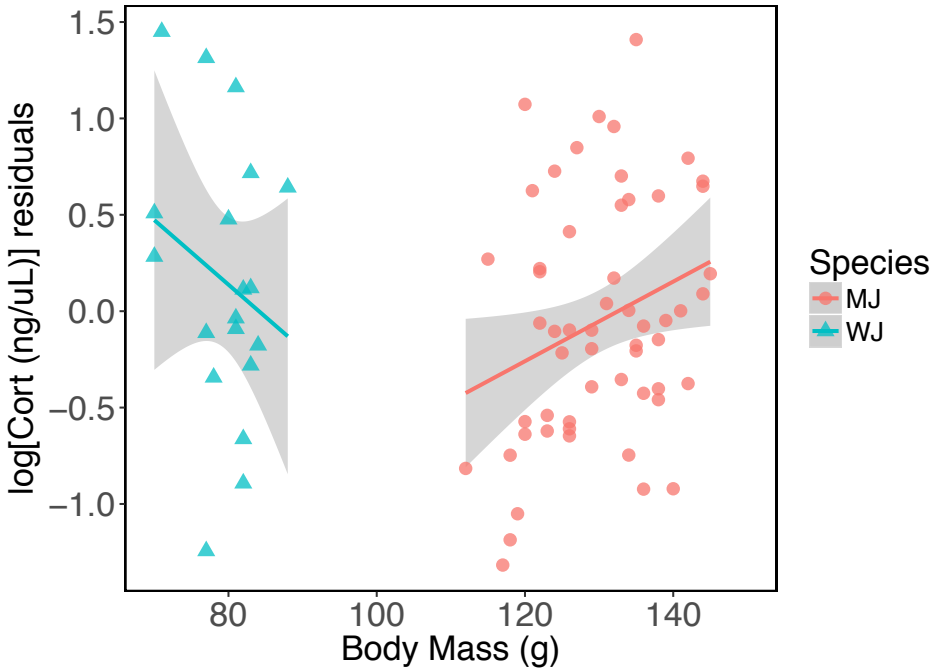

Figure 6. Comparison of body mass and residual plasma Cort concentration in Mexican jays (MJ, pink) and Woodhouse's scrub jays (WJ, blue). The grey shaded region represents +/- one standard error. There was no overall relationship between body mass and Cort concentrations *(P*=0.109) and no significant difference when comparing species by body mass (*P*=0.109).

Tables

Model	Model Subset	Sum SQ	Mean SQ	Eta2	Num DF	Den DF	F value	P value
Handling Time	Handling Time	4.082	4.082	0.115	1	73	9.517	**0.002**
MJ Helper x Breeder	Handling Time	1.47	1.47	0.102	1	41	3.789	0.058
	Breeding Status	0.985	0.985	0.053	1	41	2.54	0.118
	Sex	0.005	0.005	0.003	1	41	0.012	0.912
	Breeding Status x Sex	0.186	0.186	0.01	1	41	0.48	0.492
MJ Age	Handling Time	2.014	2.014	0.109	1	51	5.213	**0.026**
	Age	1.226	1.226	0.052	1	51	3.175	0.08
	Sex	0.177	0.177	0.017	1	51	0.459	0.501
	Age x Sex	0.262	0.262	0.011	1	51	0.678	0.413
MJ Females x Males	Handling Time	2.014	2.014	0.086	1	53	5.037	**0.029**
	Sex	0.177	0.177	0.007	1	53	0.444	0.507
MJ Body Mass x Sex	Handling Time	2.014	2.014	0.074	1	51	5.323	**0.025**
	Body Mass	1.596	1.596	0.0005	1	51	4.22	**0.045**
	Sex	0.177	0.177	0.068	1	51	0.469	0.496
	Body Mass x Sex	0.297	0.297	0.012	1	51	0.785	0.379
MJ x WJ	Handling Time	3.568	3.568	0.103	1	72	8.498	**0.005**
	Species	0.016	0.016	0.017	1	72	0.04	0.842
Seasonality (MJ and WJ)	Handling Time	2.047	2.047	0.035	1	70	5.197	**0.025**
	Species	1.046	1.046	0.036	1	70	2.655	0.107
	Day of Year	4.325	4.325	0.076	1	70	10.978	**0.001**
	Species x Day of Year	0.395	0.395	0.011	1	70	1.003	0.32
Body Mass (MJ and WJ)	Handling Time	4.082	4.082	0.099	1	70	10.011	**0.002**
	Species	0.621	0.621	0.043	1	70	1.524	0.221
	Body Mass	1.073	1.073	0.03	1	70	2.633	0.109
	Species x Body Mass	1.071	1.071	0.03	1	70	2.628	0.109

Table 1. ANCOVA results for residual plasma Cort comparisons for Mexican jays (MJ) and Woodhouse's scrub jays (WJ), with Eta^2 (η^2). Bolded values are significant (P<0.05).

Model	Hedge's g	DF	Lower 95% CI	Upper 95% CI
MJ Helpers vs. Breeders	0.362	41	-0.237	0.962
MJ <2 yr vs. >2yr	0.328	51	-0.213	0.871
MJ Females vs. Males	0.171	51	-0.713	0.371
MJ vs. WJ	0.358	72	-0.174	0.89

Table 2. Hedge's g effect sizes and 95% confidence intervals (CI) for comparisons of mean log-transformed Cort comparisons between groups.

CHAPTER THREE

Do nonapeptides regulate parental care depending on experience in zebra finches?

Abstract: The nonapeptide neurohormones regulate parental behaviors in a diverse array of vertebrates. However, it remains unclear how these neurohormones regulate parental care among birds, especially those which exhibit biparental care, common across birds, or whether hormonal effects are contingent on a bird's previous experience as a parent. I measured the effects of nonapeptides on parental behaviors by injecting, over three treatment days, a short-acting oxytocin receptor antagonist (OTA) or a saline control into breeding pairs of zebra finches (*Taeniopygia guttata*) that either did or did not have previous parental experience. I then compared how the duration and/or frequency of parental behaviors changed over the five days of observation (including one day before and two days after injections were administered). To compare treatment effects on parental outcomes, I also measured chick growth and mortality rates for each pair. OTA and experience significantly affected the amount of time birds spent nest guarding, with inexperienced birds receiving the OTA increasing nest guarding relative to inexperienced controls or experienced OTA birds. Chicks reared by parents that received the OTA had significantly lower growth rates than chicks reared by control parents and, among experienced birds, higher mortality relative to control birds. Together, these results provide some support for the hypothesis that nonapeptides play a role in regulating parental outcomes and some parental behaviors in both experienced and inexperienced zebra finches.

CHAPTER 3: Nonapeptides, Experience, and Parental Care in Zebra Finches

Introduction

Parental care is widespread throughout the animal kingdom, evolving multiple times independently among insects and other invertebrates, fishes, reptiles, mammals, amphibians, and birds (Crump 1996, Taborsky 2001, Cockburn 2006, Langkilde et al. 2007, Smith et al. 2012a, Lind et al. 2017). In animals that exhibit parental care, it is vital to offspring survival (Clutton-Brock 1991). However, relatively little is known about the neuroendocrine basis for parenting behaviors in many key taxa, which limits understanding of how parental care evolves. Birds perform more parental care than any other animal group, with 98% of species providing some type of parental care and 80% providing biparental care (Cockburn 2006). As nonapeptides (oxytocin, vasopressin, and their non-mammalian homologs) are associated with a wide range of social behaviors across vertebrates (Donaldson and Young 2008, Choleris et al. 2013), including parental care (Bosch and Newmann 2012, Schulte and Summers 2017), they are candidate neurohormones for regulating parental care in birds.

Alternatively, it is possible that nonapeptide regulation of parental behaviors, which is established in some mammal species (Bosch and Newmann 2012, Schulte and Summers 2017), does not occur in most birds. Although the nonapeptides share a common ancestral form among vertebrates (Donaldson and Young 2008), because parental care behavior independently evolved multiple times in vertebrates (fish-Gross 2005; birds-Cockburn 2006; mammals-Clutton-Brock 1991), nonapeptides may have been differentially co-opted to affect parental behavior among the diverse groups (Goodson and Thompson 2010). Differences in the presence and density of OT and AVP receptors within the brain among vertebrate taxa potentially underlie unique

responses to nonapeptides—even among closely related taxa. Comparative studies across mammals have found significant species differences in the patterns of OT and AVP receptors in the brain (see Young 1999). If true, the role nonapeptides play in regulating parental care in one species might not accurately predict its role across other species displaying parental care (Insel et al. 2001). Thus, real uncertainty exists over whether nonapeptides regulate parental care among different taxa, which can only be resolved by novel experimental investigations.

Previous experimental manipulations of nonapeptides provides some examples of unique mechanisms between closely related taxa and other examples of conserved nonapeptide regulation between more distantly related taxa. For example, administration of an oxytocin (OT) or arginine vasopressin (AVP) receptor antagonist has been shown to significantly reduce maternal care in female rats (OT- van Leengoed et al. 1987; AVP-Bosch and Neumann 2008). ICV infusions of OT or AVP also increase maternal care in rats (OT-Pedersen and Prange 1979, Bosch and Neumann 2008; AVP-Bosch and Neumann 2008). However, in mice (*Mus musculus*), OT-knockout females and females receiving an OT receptor antagonist (OTA) displayed maternal care behaviors identical to control females with the exception of lactation (OT-knockout-Nishimori et al. 1996, Young et al. 1996; OTA-Insel et al. 2001). Illustrative of the lack of phylogenetic conservatism in nonapeptide regulation of parental behavior, sheep exhibit a similar mechanism to rats (but not mice), with ICV infusions of OT inducing maternal care in nulliparous or in post-partum ewes that were non-maternal (Keverne et al. 1983, Kendrick et al. 1987).

Though less well studied than in mammals, research outside of mammals

supports the hypothesis that nonapeptides can regulate parental care behaviors in other vertebrate taxa as well. An arginine vasotocin (AVT, AVP homolog) receptor antagonist significantly reduced maternal care in pigmy rattlesnakes (*Sistrurus miliarius*) (Lind et al. 2017) and reduced parental care in both males and females in the biparental poison frog (*Ranitomeya imitator,* Schulte and Summers 2017). In birds, mesotocin (MT, OT homolog) plays a role in regulating brooding behaviors in both turkeys (*Melagris gallopavo*) and chickens (*Gallus domesticus*). Turkey hens given ICV infusions of an OT receptor antagonist significantly reduced their likelihood of brooding chicks (Thayananuphat et al. 2011). In chickens, presence of MT and MT neurons are correlated with maternal nest attendance and chick care (Chokchaloemwong et al. 2013). In songbirds, administration of an OT receptor antagonist reduced nest-building behaviors in female but not male zebra finches (*Taeniopygia guttata*), whereas an AVP receptor antagonist reduced nest building in both sexes (Klatt and Goodson 2013b).

Although less well studied than nonapeptide regulation of maternal care, nonapeptides may also regulate paternal care behavior, though the results differ among taxa and specific nonapeptides. For example, central infusion of AVP promotes paternal care in the biparental prairie vole (*Microtus ochrogaster*) (Wang et al. 1994) and the facultatively biparental meadow vole (*M. pennsylvanicus*) (Parker and Lee 2001) and an AVP receptor antagonist inhibited paternal care in meadow voles (Parker and Lee 2001). However, though paternal behavior was inhibited in virgin male prairie voles treated with combined AVP and OT receptor antagonists, neither AVP nor OT receptor antagonists had the same effect when administered in isolation (Bales et al. 2004a). Similar results were found in fishes, where an isotocin (IT, fish oxytocin homolog)

receptor antagonist significantly reduced paternal care in both the biparental convict cichlid (*Amatitlania nigrofasciata*) (O'Connell et al. 2012) and the paternal-care anemonefish (*Amphiprion ocellaris*) (DeAngelis et al. 2017). In contrast, male anemonefish receiving an AVT V1a receptor antagonist significantly increased paternal care (DeAngelis et al. 2017). Therefore, the nonapeptides are likely involved in paternal care across vertebrates, though the exact regulatory mechanisms differ among species.

Another factor affecting parental care is experience. In mammals previous maternal care bouts lead to greater future parental competency, allowing mothers to respond correctly to offspring cues even without hormonal priming from pregnancy, a phenomenon termed maternal memory (Corter and Fleming 1995, Holman and Goy 1995, Dwyer and Lawrence 2000). Primiparous rats given an OT receptor antagonist during postpartum days 6-7 significantly reduced oral grooming of pups and upright posturing over pups (Pedersen and Boccia 2003). However, rats given OT anti-sense oligonucleotides on day 18 postpartum, by which time the memory has formed, displayed no changes in maternal behavior (Giovernardi et al. 1998). These results suggest that, in rats, OT is necessary for the transition to maternal care, but not for its continued maintenance after maternal care is established (Insel et al. 2001).

Experience, independent of hormonal manipulation, affects parenting in birds as well, with chicks raised by inexperienced parents growing more slowly than chicks raised by experienced parents (kittiwakes, *Rissa tridactyla*, Coulson and Porter 1985; zebra finches, Skagen 1988). In black-browed albatrosses (*Thalasarche melanophris*), fledging success was higher in experienced pairs than those breeding for the first time (Angelier et al. 2007). Experienced zebra finches also feed chicks more than

inexperienced birds, but chick feeding was nearly eliminated regardless of experience when birds were given a drug to reduce prolactin in the three final days before hatching and first two days post-hatching (Smiley and Adkins-Regan 2018a). It is possible that parental experience may not be regulated the same way in birds as in mammals. It is also possible that disrupting normal hormonal regulating right before and immediately after chicks hatched may have disrupted the ability of experienced parents to access their parental memories. Thus, it remains unclear if nonapeptides regulate parental care behaviors in previously unstudied taxa and whether this regulation will differ according to previous parental experience. Testing this requires fully factorial manipulations of the nonapeptides, with controls, in experience and inexperienced parents.

In birds, promiscuous binding of mesotocin (avian homolog of OT) and vasotocin (avian homolog of AVP) to multiple nonapeptide receptors makes it difficult to distinguish between their effects on behavior. Similar to other vertebrates, birds have multiple vasotocin/vasopressin receptors and one OT-like receptor (Baeyens and Cornett 2006). In birds, the vasotocin (V1a and VT1) and mesotocin (OT-like VT3) receptors are widespread throughout the brain, particularly in regions associated with social behaviors (Leung et al. 2011). The VT3 receptor of zebra finches will bind both mesotocin and vasotocin (Baeyens and Cornett 2006, Leung et al. 2009). Therefore, any behavioral effects of a receptor antagonist targeting VT3 could be due to blocking mesotocin, vasotocin, or both nonapeptides.

I tested whether nonapeptides and parental experience may affect parental care behaviors in the biparental zebra finch. I administrated an oxytocin receptor antagonist (OTA) or vehicle control over multiple days to inexperienced and experienced birds and

measured the effects on parental care. I predicted that blocking the nonapeptides would reduce parental care behaviors and negatively affect chick outcomes, especially in inexperienced zebra finches.

Methods

Subjects and housing

This experiment involved the measurement of behaviors and chick outcomes for a total of 64 adult zebra finches in 32 pairs of males and females, with 16 pairs having previous experience raising offspring and 16 pairs with no previous parental. For both experience groups, 8 pairs were randomly assigned to the OTA treatment and 8 pairs to the saline control. All subjects had been bred in the lab. The experienced birds had been freely allowed to choose their partners prior to the start of the study and had successfully raised at least one clutch of offspring together. Inexperienced birds were kept in same-sex cages prior to the start of the experiment. To allow inexperienced birds to choose their partners, birds were placed in mixed-sex cages (4 inexperienced males and 4 inexperienced females) without nest boxes and allowed to pair naturally. Pairs were determined based on time spent clumping together, allopreening, and copulations. Once paired, inexperienced pairs were placed in breeding aviaries with experienced pairs so that each aviary had four total pairs. To control for any aviary effects, pairs were randomly selected and assigned to either control or treatment group and each aviary included one experienced OTA pair, one inexperienced OTA pair, one experienced control pair, and one inexperienced control pair. During the experiment, I allowed pairs to continue to try to breed until all other pairs in their aviary had completed the experiment, but pairs that did not lay eggs or did not have any eggs hatch were

excluded from the study. The final sample sizes were as follows: experienced control (EXP-C), N=10 (N=5 female-male pairs); inexperienced control (INEXP-C), N=10 (N=5 female-male pairs); experienced OTA (EXP-OTA), N=12 (N=6 female-male pairs); inexperienced OTA (INEXP-OTA), N=14 (N=7 female-male pairs).

All aviaries had dimensions of 0.94 m by 0.76 m by 0.94 m and were equipped with seed, grit, cuttlebone and water ad libitum as well as four empty nest boxes with perches on the front of each nest box, and coconut fiber nest material in the cage. All subjects were housed in a temperature and humidity controlled room on a 14:10 light:dark schedule for the duration of the experiment. Each individual had four unique leg bands; three colored and one silver ID band with an individualized ID code. All aviaries were located within the same room. The Cornell University IACUC approved all methods and procedures of the study.

OTA injections

Each bird in the experimental group was given a 0.05 ml intramuscular injection into the pectoral muscle of 5 µg OTA ([d(CH_2)$^1{}_5$, Tyr(Me)^2, Thr^4, Orn^8, des-Gly-$NH^9{}_2$]-Vasotocin trifluoroacetate salt, Bachem) dissolved in 0.9% saline. In rats, this antagonist is 18 times more potent as an OT receptor antagonist than a V1a receptor antagonist (Manning et al. 2008) and has been highly effective in altering rat social behaviors (Neumann et al. 2003, 2006). This antagonist has also effectively disrupted social behaviors in zebra finches (Goodson et al. 2009, Pedersen and Tomaszycki 2012, Klatt and Goodson 2013a, 2013b). When testing the effects of this OTA on pair formation in zebra finches, 1 µg, 5 µg, and 10 µg were all shown to the formation of pair bonds and

the 5 μg dose specifically disrupted more pairing behaviors than either the 1 μg or 10 μg doses (Pedersen and Tomaszycki 2012). As there were no dose dependent effects of this OTA on whether birds paired (Pedersen and Tomaszycki 2012), I chose to inject the pairs with their medium dosage of 5 μg OTA. Control subjects were injected with the same volume of 0.9% saline. Both males and females within a pair were given the same treatment. Animals were only injected once per day for three days and the effects of each injection likely did not last the entire 24-hour period of each day (Pedersen and Tomaszycki 2012).

Behavioral recording and measurement

In order to determine the effects of OTA on parental care, I recorded each pair for five days beginning on the second day after the first chick hatched: Pre-treatment (Day 0, no injection, first chick is two days old), three consecutive days of injections (Days 1-3), and Post-treatment (Day 5, no injection, 48 hours after the third injection, first chick is seven days old). On Days 1-3, birds were caught and injected with either OTA or saline control and then placed back within their home cage. On Pre-treatment and Post-treatment days, birds were also caught and briefly held to create equivalent handling stress before being placed back within their home aviary. After being placed back within the aviary, each pair was given 15 minutes to allow the OTA to take effect, well within the time range (10-30 minutes) found to be effective in previous studies (Samuelsen and Meredith 2011, Pedersen and Tomaszycki 2012). Pairs were then video recorded for 60 minutes. For all recordings, the video camera was placed in front of the home aviary, focused on the nest of the focal pair. For all groups, recordings

occurred between 8 am and 12 pm and time of day was kept consistent for each pair throughout the experiment.

Videos were scored separately for each male and female by a single trained coder who was blind to treatment. Parenting behaviors (see Table 3) were scored based in part on previous studies of parental care in zebra finches (ten Cate 1982, Hall et al. 2015, Mariette and Griffith 2012). Specifically, I scored total time a bird spent within the nest (time in nest), time spent perched in front of the nest (nest guarding), and time spent manipulating nest material around the nest opening (nest maintenance) (Table 1). Though I also monitored how frequently nest material was brought to the nest and how often focal birds chased other birds away from the nest when nest guarding, these behaviors were too rare to include in our behavioral analyses. Some videos were excluded due to technical failure of the camera to record or computer equipment to save the video. Therefore, the following total numbers of videos were scored for each sex per group: females (EXP-C, N=21; INEXP-C, N=25; EXP-OTA, N=29; INEXP-OTA, N=34), males (EXP-C, N=21; INEXP-C, N=25; EXP-OTA, N=29; INEXP-OTA, N=34). For parental care behaviors, one nest only had video for two days (1 EXP-C-Pre-Treatment and Day 1) and three nests only had video data for four days (1 EXP-C-Pre-Treatment, Day 1, Day 3, Post-Treatment; 1 EXP-OTA-Day 1, Day 2, Day 3, Post-Treatment; 1 INEXP-OTA- Pre-Treatment, Day 1, Day 2, Post-Treatment). Missing video data was excluded from all behavioral analyses.

Chick effects

As I did not have cameras inside the nest, I could not distinguish between time

spent brooding or feeding chicks when a bird was in the nest. Therefore, throughout the experiment, I also measured the mass of each chick daily as a proxy for parental feeding rates. I used this data to fit logistic growth curves for each chick (Initial body mass and Max chick growth rate). I also monitored how many chicks died during the study period (Chick mortality). Chick data was obtained for all nests in all treatment groups.

Data analysis

Behavioral Effects

I used linear mixed-effect models to determine how treatment and experience affected the change in the time invested in the three parenting behaviors: time in nest (time spent in the nest for each bird), nest guarding time (time spent on the perch outside the nest), and nest maintenance time (time spent manipulating nest material at the opening of the nest). All behaviors were analyzed at the level of the individual bird. To address potential non-independence of an individual's behavior due to how their partner behaved or what cage they were in, Bird ID (unique code for each bird) was nested within Pair ID (unique identifier for each pair) and Pair ID was nested within Cage ID (unique code for each cage) as random effects for all models. The data was not transformed. All statistical tests were performed in R (version R 3.4.0).

Sex was initially included as a fixed effect in the linear mixed effects models both as a main effect and in the four way interaction of Sex x Treatment x Experience x Day, but was never a significant term for any of the behaviors, as determined by post-hoc contrasts, and was subsequently removed from all statistical models. After removing

sex, all linear mixed effects models were run with one fixed effect: a three-way interaction of Treatment x Experience x Day (Table 4). Because treatment and experience effects on parental behaviors were measured as the change in behavioral duration over time, Treatment and Experience main effects were omitted from these models; corrections for the difference in the initial duration of behaviors (on the pre-treatment Day 0) among groups and individual birds were made using the nested random-intercept effects described above. Note that including one fixed interaction term means that, using ANOVA, this term could be significant without implying that the slopes of the individual Treatment x Experience groups differ significantly from one another; thus, ANOVAs were not used to evaluate the significance of Treatment and Experience effects on parental behaviors. Rather, post-hoc contrasts of the individual slopes were used to test for the existence of Treatment and Experience effects on the change in behavioral duration over the course of the experiment.

To determine the significance, sign, and magnitude of Treatment and Experience effects, I used the “lsmeans” package in R to contrast the slopes of the duration of behavior vs Day (ranging from 0-5), as measured over a 60 minute observation period, between the following groups: (1) EXP-CON vs EXP OTA (experienced treatment effect); (2) INEXP-CON vs INEXP-OTA (inexperienced treatment effect); (3) EXP-CON vs INEXP-CON (experience effect in the control); and (4) EXP-OTA vs INEXP-OTA (experience effect in the OTA treatment). A Tukey-correction for four contrasts was applied to the p-values; contrasts were considered significant when $p<0.050$ (Table 5).

Following Kline (2004), non-standardized effect sizes in their original units are reported, rather than approximations of effect size statistics such as Cohen’s *d*, due to

the difficultly of estimating the pooled variance of groups of semi-independent measurements in models with complicated random-effect structures (*see also* Nakagawa and Cuthill 2007). Thus, the effect size is a measurement of the difference in slopes of the change in the behavioral duration vs. day between experience levels in the same treatment (2 contrasts) and between treatments of the same experience level (2 contrasts) (Table 5). For example, for a contrast of behavioral duration between the control and OTA treatments, an effect size of 100 (seconds/day) means that, on average, the birds in the control treatment increased the behavior by an additional 100 seconds/day relative birds in the OTA treatment.

In order to assess uncertainty around the treatment effects, 95% confidence intervals around non-standardized effect sizes (also in original units of seconds/day) were generated using the "lsmeans" package in R. Similar to the reported p-values, a Tukey-correction for 4-contrasts was also applied to the width of the reported confidence intervals (Table 5). In general, when the 95% confidence interval does not overlap with zero, the contrast is significant at $p<0.05$.

Chick Logistic Growth Rate

I estimated parameters by fitting a logistic growth rate equation for each chick (methods from Royle et al. 2006), such that

$$M=A/(1+\exp(-Kt+B)),$$

where M is chick body mass (in grams), A is the asymptotic body mass (an estimate of adult body mass), K is the logistic growth constant, t is time in days (day of birth is day 0), and the initial body mass (when $t=0$) is equal to the expression $A/(1+\exp(B))$. The

logistic growth function reaches its maximum rate of $AK/4$ when $t=B/K$, which is also when chicks mass is equal to half the asymptotic body mass ($M=A/2$).

This model predicts natural zebra finch chick growth, as chick growth follows a logistic growth curve when measured from hatching through fledging in the same lab when receiving the same food and under the same rearing conditions (Banerjee and Adkins-Regan 2014). Although it is possible to project asymptotic (adult) body mass by fitting a logistic curve to the chick mass data, because chicks were measured for only the first seven days post-hatch, these projections cannot be made with high confidence. Based on the findings of Banerjee and Adkins-Regan (2014), asymptotic body mass is not reached until 30-40 days post-hatch. Therefore, I analyzed only the estimates of initial body mass and max growth rate for the chicks in each experimental group.

For each chick, I estimated the parameters A, B, and K using the nonlinear least squares method (nls function) in the R base statistics package. I omitted data from chicks that died during the experiment from our analysis. It was not possible to estimate parameters for all chicks, as some lacked observations from a sufficient number of days for models to converge on stable parameter estimates. Overall, chick parameters were estimated for 36 out of a total of 54 chicks that were alive at the end of the experiment (for a proportion of total living chicks with initial and max growth rates estimates of 8/15 for EXP-C, 8/11 for EXP-OTA, 5/9 for INEXP-C, and 15/19 for INEXP-OTA).

Statistics For Logistic Growth Rate

For the logistic growth rate parameters of birth mass ($A/(1+\exp(B))$) and maximum growth rate ($AK/4$), I ran linear mixed effect models with random-intercepts

calculated for Pair ID nested within Cage ID (because each chick has only one set of fitted parameters, there was no repeated measurement correction for individual chicks). Each model initially included treatment, experience level, and treatment x experience interaction terms as fixed effects. I removed the interaction term from the final models when it was insignificant, as determined using ANOVA ($p>0.05$).

Similar to the effect size and confidence intervals reported for comparisons of changes in behavioral duration over time among Treatment x Experience groups, 95% confidence intervals around non-standardized effect sizes for the difference in mean initial birth weight and mean maximum growth rate (in units of grams and g/day, respectively) were generated using the "lsmeans" package in R. Because there was never a significant Treatment x Experience interaction effect, only two post-hoc contrasts were performed: (1) Control vs OTA birds (averaged across experience levels) and (2) EXP and INEXP birds (averaged across treatment levels). A Tukey-correction for 2-contrasts was also applied to the width of the reported confidence intervals; the null hypothesis of equal means was rejected for p values <0.05.

Total Number of Chicks and Chick Mortality

I ran linear mixed effect models for the total number of chicks hatched during the experiment to each pair, with experience level, treatment, and experience x treatment interactions as fixed effects and Cage ID as a random intercept. Significant effects were determined using ANOVA at $p<0.05$. For chick mortality, I tested the hypothesis of unequal numbers of dead chicks in each treatment x experience combination by performing four Fisher's exact test contrasts, two each between treatments (EXP-C vs.

EXP-OTA and INEXP-C vs. INEXP-OTA) and between experience levels (EXP-C vs. INEXP-C and EXP-OTA vs. INEXP-OTA). I rejected the null hypothesis at $p<0.05$ after performing a Bonferroni correction for multiple contrasts.

Results

Treatment x Experience Parental Behaviors

Time spent in nest: Total time spent in the nest significantly declined across the days for EXP-OTA (-270.83 ± 58.42 s/day [effect size in slope ± SE for all behaviors], $T_{31.75}$=-4.636, P=0.00005), INEXP-OTA (-148.42 ± 54.94 s/day, $T_{31.4}$=-2.701, P=0.011), and INEXP-C (-177.32 ± 62.67 s/day, $T_{29.54}$=-2.829, P=0.008) birds, but did not significantly change for EXP-C birds (-82.99 ± 68.27 s/day, $T_{35.05}$=-1.216, P=0.232). However, all post-hoc comparisons of the slopes of total time spent in the nest vs. day were insignificant (P>0.05 for EXP-C vs. EXP-OTA, INEXP-C vs. INEXP-OTA, EXP-C vs. INEXP-C, EXP-OTA vs. INEXP-OTA) (Table 5) (Fig. 7).

Nest guard: Total time spent nest guarding significantly increased across the days for INEXP-OTA birds (28.863± 7.498 s/day, $T_{22.96}$=3.583, P=0.001), but did not significantly change for EXP-OTA, EXP-C, and INEXP-C birds (all P >0.05) (Table 4). Post-hoc contrasts of slopes of time spent nest guarding were significantly different for INEXP-C vs. INEXP-OTA (-26.524 ± 9.647 s/day, $T_{25.13}$=-2.749, P=0.042) and EXP-OTA vs. INEXP-OTA (-24.756± 9.232 s/day, $T_{26.26}$=-2.682, P=0.049) with time spent nest guarding declining for INEXP-C and EXP-OTA compared to INEXP-OTA (Fig. 8). However, post-hoc contrasts of EXP-C vs. EXP-OTA and EXP-C vs. INEXP-C were insignificant (P >0.05) (Table 5) (Fig. 8).

Nest maintenance: Total time spent performing nest maintenance significantly declined across the days for EXP-OTA (-7.695± 3.137 s/day, $T_{68.19}$=-2.539, P=0.013), INEXP-OTA (-6.412± 3.073 s/day, $T_{60.85}$=-2.087, P=0.041), and EXP-C (-7.134± 3.489 s/day, $T_{91.35}$=-2.045, P=0.043), whereas the slope of time spent performing nest maintenance vs. day did not significantly differ from zero for INEXP-C (-2.114± 3.264 s/day, $T_{75.88}$=-0.648, P=0.519). However, all post-hoc comparisons of the slopes of total time spent performing nest maintenance vs. day were insignificant (P>0.05 for EXP-C vs. EXP-OTA, INEXP-C vs. INEXP-OTA, EXP-C vs. INEXP-C, EXP-OTA vs. INEXP-OTA) (Table 5) (Fig. 9).

OTA and Experience on chick mass, growth rates, and mortality

For initial body mass and maximum growth rate, there were no significant treatment by experience interactions; interaction terms were dropped during model selection. The initial (intercept) body mass of chicks did not differ between chicks with control or OTA parents (Mean difference: 0.028± 0.065 g, $F_{12.65}$=0.354, P=0.562) or experience groups (Mean difference: 0.065± 0.067 g, $F_{15.85}$=0.585, P=0.455) (Fig. 10A). Maximum chick growth rates were significantly higher in the control relative to the OTA treatment (Mean difference: 0.793± 0.183 g/day, $F_{15.88}$=10.986, P=0.004), though there were no differences between experience levels (Mean difference: -0.424± 0.188 g/day, $F_{16.61}$=2.955, P=0.104) (Fig. 10B).

Overall, 75 chicks hatched across the four treatment groups (EXP-C: 15 total, 3 mean ± 0.547, 2-5 range; EXP-OTA: 20 total, 3.333 mean ± 0.843, 1-7 range; INEXP-C: 16 total, 3.2 mean ± 0.374, 2-4 range; INEXP-OTA: 24 total, 3.428 mean ± 0.528, 1-5

range). The number of chicks that hatched per pair during the experiment did not significantly differ by treatment or experience (all $P>0.05$) (Table 6). Across the four groups, 21 chicks died during the experiment, with a proportion of dead chicks of 0/15 for EXP-C, 9/20 for EXP-OTA, 7/16 for INEXP-C, and 5/24 for INEXP-OTA. Fisher's exact tests with a Bonferroni correction found EXP-C had significantly fewer chicks die than EXP-OTA ($P=0.02$) or INEXP-C *(*$P=0.02$) (Fig. 11). There were no significant differences in chick mortality between EXP-OTA and INEXP-OTA *(*$P=0.199$) and INEXP-C and INEXP-OTA ($P=0.199$) (Fig. 11).

Confidence Intervals

For time spent nest guarding, the significantly different differences in slopes were as follows: INEXP-C vs. INEXP-OTA (Δslope: -26.524, 95% confidence interval, CI, range: -52.412 to -0.636 seconds/day) and EXP-OTA vs. INEXP-OTA (Δslope: -24.756, 95% CI: -49.53 to -2.682), all in units of seconds/trial day over a 60-minute observation period. By contrast, for time spent nest guarding the differences in slope for EXP-C vs. INEXP-C and EXP-C vs. EXP-OTA were not significant ($P>0.05$, CI range included 0, Table 5). Although not statistically significant, the largest differences in slope were observed for time in the nest: EXP-C vs. EXP-OTA (Differences in slope-DSlope: 187.842, 95% CI range: -21.995 to 397.679), INEXP-C vs. INEXP-OTA (DSlope: -28.897, 95% CI: -218.169 to 160.373), EXP-C vs. INEXP-C (DSlope: 94.327, 95% CI: -124.706 to 313.361), and EXP-OTA vs. INEXP-OTA (DSlope: -122.412, 95% CI: -302.835 to 58.01). The differences in slopes and 95% CI for nest maintenance were small (Δslopes ranged between -5.037 and 0.855) and not significantly different (Table

5).

The confidence intervals for the significantly different difference in means for chick max relative growth rate were for Treatment (Mean difference: 0.793± 0.183 g/day, 95% CI range: 0.364 to 1.223). By contrast, the differences in mean for Experience for max growth rate was not significant (P>0.05, CI range included 0, Table 7). The differences in means and 95% CI for treatment and experience for initial body mass were small (Mean difference ranged between 0.028 and 0.065) and not significantly different (Table 7).

Discussion

I tested whether administration of OTA could disrupt parental care in zebra finches and if previous experience as parents would ameliorate or prevent this disruption. There was a significant effect of experience and OTA on nest guarding, with nest guarding in INEXP-C and EXP-OTA birds declining relative to INEXP-OTA birds. There was no effect of the antagonist or experience on nest maintenance or total time spent in the nest. By contrast, the OTA treatment did have a negative effect on chick outcomes. Chicks reared by parents that received the OTA had significantly lower maximum growth rates than chicks reared by control parents and, among experienced birds, higher mortality relative to birds with control parents. Therefore, though the nonapeptides do not seem to regulate some of the parental behaviors examined here, they do play an important role in chick outcomes.

The finding that OTA did not affect time spent in the nest (which would include chick brooding) contrasts with previous results reported for chickens (*Gallus*

domesticus) and turkeys (*Meleagris gallopavo*), where brooding is correlated with elevated levels of MT neurons (chickens, Sinpru et al. 2018) and ICV infusions of an OTA significantly reduced chick brooding (turkeys, Thayananuphat et al. 2011). One potential explanation for the observed negative result for OTA effects on time spent in the nest could be differences in the hormones that regulate brooding among species. Songbirds such as zebra finches and galliform birds (chickens and turkeys) are only very distantly related, belonging to different orders.

Methodological issues specific to the route of delivery and specificity of the receptor antagonist could also underlie the lack of an OTA effect on time spent in the nest. For example, recent work on pair formation in zebra finches found opposite effects of the same OTA on male singing depending on whether the antagonist was delivered through peripheral injections (the method used in this study) or ICV infusions (peripheral reduced song, Pedersen and Tomaszycki 2012; ICV increased song, Klatt and Goodson 2013a). Given that the route of delivery of the same OTA can result in qualitatively different outcomes, it remains unclear how ICV infusions of OTA would have affected time in the nest – as well as the other behaviors studied here.

Additionally, for comparisons between this study and a study of nonapeptide regulation in reptiles, it is necessary to consider the specificity of the experimental receptor antagonists. The OTA used in this study is specific to the OT-like VT3 receptor (Manning et al. 2008), but not to the AVT receptor V1a. Thus, whereas the AVT receptor antagonist has been shown to significantly reduce time spent with offspring in rattlesnakes (*Sistrurus miliarius*; Lind et al. 2017), this study antagonized a receptor that would not have been affected by the OTA used here. Thus, nonapeptides could

regulate of zebra finch parental behaviors performed in the nest (brooding and chick feeding inclusive) through AVT at the V1a receptor, which was unaffected in this study.

There was a significant treatment effect of OTA on nest guarding (perching in front of the opening to the nest) in inexperienced but not experienced birds. INEXP-OTA birds increased nest guarding compared to both EXP-OTA and INEXP-C groups. Results of research on the role of nonapeptides in regulating nest/egg guarding vary across vertebrates. Male three-spined sticklebacks (*Gasterosteus aculeatus*) have higher levels of AVT in the brain during their paternal phase (including when egg guarding) compared to any other reproductive stages (Kleszczyńska et al. 2012). However, neither increasing nor decreasing circulating levels of MT (through injections of MT or its antagonist, respectively) had any effect on egg guarding behavior in females or males of the biparental poison frog (*R. imitator*) (Schulte and Summers 2017). The role experience plays in amount of time an individual spends nest guarding also varies across species. Previous work on eastern kingbirds (*Tyrannus tyrannus*) found that males with previous parental experience performed more nest guarding than inexperienced birds (Woodard and Murphy 1999); although in this case the investigators contrasted total time spent nest guarding, and not the change in nest guarding in response to hormonal manipulation. Therefore, the effect of experience and the nonapeptides on time spent nest guarding may be species specific and affected by factors including the presence of nest predators (Komdeur and Kats 1999) and egg dumping (Gowaty et al. 1989), which were not factors in this study.

The time spent performing nest maintenance significantly declined over the course of the experiment for three groups (EXP-C, EXP-OTA, INEXP-OTA), but the

slopes of these declines did not differ from one another in pairwise contrasts. This decline in nest maintenance is likely due to a natural shift in care behaviors rather than to experience or OTA. Zebra finches spend less time in the nest after post-hatch day 5 (ten Cate 1982) and significantly increase their feeding rates as chicks grow (Mainwaring et al. 2011). Reducing time in the nest and spending more time collecting food and feeding chicks would reduce the amount of time parents could spend performing nest maintenance. Previous work suggests that nonapeptides regulate nest building in zebra finches; with injection of an OTA resulting in reduced nesting behavior in female zebra finches and injection of a specific AVT receptor antagonist (V1aR) modestly reduced nesting behaviors in both female and male zebra finches (Klatt and Goodson 2013b). However, as I did not start the injections until the third day after the first chick hatched and only used an OTA and not a specific V1a receptor antagonist, nests were already well established before injections, which may have decoupled nest maintenance behaviors from the nonapeptides.

Though I found no effect of OTA on time spent in the nest or nest maintenance, the OTA may be effective at higher or lower doses, as nonapeptide regulation of behavior tends to be dose-dependent. For example, low, but not high, doses of oxytocin facilitate social recognition in rats (Popik et al. 1992, Benelli et al. 1995). Female prairie voles also respond to oxytocin in a dose-dependent manner, with high but not low doses of oxytocin reducing female partner preference and likelihood of forming pair bonds (Bales et al. 2007). Administration of an OTA to zebra finches also had a dose-dependent effect on specific pair formation behaviors, with the medium (5μg) but not low (1μg) or high (10μg) OTA dose significantly reducing the time females spent in the

nest box with males (Pedersen and Tomaszycki 2012). As the present study used only a single dosage (5μg), future work on the role of nonapeptides in regulating parental care in birds should include different doses of both the nonapeptides and nonapeptide antagonists.

Whereas I am only able to reject the null-hypothesis for the OTA effects on one parental behavior – nest guarding – the OTA effects on chick outcomes are both consistent and statistically significant. Chicks with parents that received the OTA had lower maximum growth rates than control chicks, but there was no difference in growth rates associated with previous parental experience. The lack of an experience effect on chick growth rate is inconsistent with previous work, where chicks raised by inexperienced parents grew more slowly than chicks raised by experienced parents (zebra finches, Skagen 1988; kittiwakes, *Rissa tridactyla*, Coulson and Porter 1985). Length of study may explain these discrepancies, as my study took place over seven days whereas Skagen (1988) and Coulson and Porter (1985) measured chick body mass through fledging. However, this shorter time span was sufficient to detect the effects of OTA on chick growth rates. The antagonist may have reduced motivation to interact with chicks or perform chick care behaviors such as chick feeding. This result is consistent with previous research on oxytocin, where alloparental meerkats (*Suricata suricata*) significantly increased their rates of pup feeding when injected with oxytocin (Madden and Clutton-Brock 2010). In zebra finches, chick growth rate is the most accurate way to estimate how much food each chick receives from their parents. Recent work on wild zebra finches has found that the quantity of food transferred per regurgitation was not correlated with the duration of each regurgitation or with chick

begging behavior (Gilby et al. 2011). Therefore, the lower chick growth rates here likely indicated reduced parental provisioning by pairs receiving the OTA.

Lower feeding rates (as measured indirectly by chick growth rates) may have led to an increase in chick mortality for experienced antagonist birds and inexperienced control birds. As zebra finch parents increase their feeding rates as chicks gain body mass (Mainwaring et al. 2011), chicks in nests with lower feeding rates would gain weight more slowly, leading to a positive feedback cycle of reduced feeding rates and higher risk of chick mortality. Chick mortality is not uncommon in zebra finches and generally increases in wild zebra finches as brood size increases (Gilby et al. 2011). I observed a significant OTA by experience interaction in which EXP-C had fewer chicks die compared with both EXP-OTA and INEXP-C birds in the absence of significant differences in the number of chicks hatched. Nine out of twenty chicks died in the EXP-OTA group and seven out of sixteen chicks died in the INEXP-C group, whereas all fifteen chicks hatched in the EXP-C group survived over the course of the experiment.

Chick mortality might also have contributed to slower chick growth rates in surviving chicks. Offspring stimuli can lead to the expression of parental behaviors, even among animals that are not currently breeding (Cohen and Bridges 1981, Scanlan et al. 2006). In birds, exposure to chicks can induce parental care in zebra finches that have eggs but have not yet hatched chicks (Smiley and Adkins-Regan 2018b) and in chickens (Richard-Yris et al. 1987) and red-winged blackbirds (Agelaius phoeniceus) (Holcomb 1979). Motivation to parent offspring can be so powerful as to lead to spontaneous adoption of non-related chicks in herring gulls (*Larus argentatus*) (Graves and Whiten 1980). In my experiment, nests with higher chick mortality would have had

fewer begging chicks, reducing overall feeding stimuli, leading to the slower surviving chick growth rates observed in the OTA groups.

Though the OTA significantly affected chick mortality and growth rates, the lack of support for both an effect of experience and OTA on parental behaviors may be a product of insufficient sample size. For example, in experienced birds, the magnitude of the estimated difference in slopes between OTA and control birds for the change in time spent in the nest over the experimental period was large relative to the other slope contrasts. The difference amounted to OTA birds sustaining greater than a 3-minute decline in the time spent in their nests per day for 5 days, as observed over a 60-minute observation period, relative to control birds. However, although such large behavioral differences could conceivably have contributed to the different parental outcomes – particularly the significantly lower chick-growth rates and higher chick mortality in the OTA vs. the control treatment – this remains unclear due to the large uncertainty in the magnitude of this effect, as indicated by the wide breadth of the 95% confidence intervals. Thus, with the present data we can draw no conclusions about the relationship between this behavior and parental outcomes. Future research on nonapeptides and parental care in birds should increase the sample size, as a larger sample size would reduce uncertainty in the effect size of the OTA treatment on time spent in the nest.

Conclusions

This study demonstrates that nonapeptides and experience play a role in some parental care behaviors in zebra finches. Whereas I found no evidence that either OTA or parental experience affects time spent in the nest or nest maintenance, I did observe

a significant effect of both experience and treatment on nest guarding, with INEXP-OTA birds performing more nest guarding than INEXP-C or EXP-OTA birds. Moreover, OTA and experience affected chick outcomes, with chick growth significantly reduced in the OTA treatment and chick mortality increased in EXP-OTA and INEXP-C compared to EXP-C birds. The negative affect of OTA on chick growth rate and increase in chick mortality is consistent with OTA parents decreasing food provisioning to chicks, though I did not directly measure provisioning rates. Overall, these results suggest that nonapeptides regulate parental behaviors important to chick outcomes, even in experienced birds, though the behavioral mechanism remains unclear.

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Tables

Behavior	Description
Total brood	Total time spent within the nest box
Nest-guarding	Perching on the nest box
Nest-maintenance	Manipulation of nest material around the nest opening

Table 3. Parental care behaviors scored for zebra finches. All behaviors were scored for both sexes and for total time spent performing the behavior.

Behavior	Model Subset	Estimate	SE	DF	T value	P value	Random effects per model		
							Category	*Variance*	*SD*
Time in Nest	EXP-C x Trial day	-82.99	68.27	35.05	-1.216	0.2323	Bird ID: (Pair ID: Cage)	10656	103.23
	INEXP-C x Trial day	-177.32	62.67	29.54	-2.829	**0.0083**	Pair ID: Cage	1797	42.39
	EXP-OTA x Trial day	-270.83	58.42	31.75	-4.636	**0.00005**	Cage	3446	58.7
	INEXP-OTA x Trial day	-148.42	54.94	31.4	-2.701	**0.011**			
Nest guard	EXP-C x Trial day	3.084	9.628	28.625	0.32	0.751	Bird ID: Cage	172.64	13.139
	INEXP-C x Trial day	0.339	9.098	24.357	0.037	0.97	Pair ID: Cage	1307.35	36.157
	EXP-OTA x Trial day	2.107	8.581	23.084	0.246	0.808	Cage	95.68	9.782
	INEXP-OTA x Trial day	26.863	7.498	22.958	3.583	**0.001**			
Nest maintenace	EXP-C x Trial day	-7.134	3.489	91.35	-2.045	**0.043**	Bird ID: (Pair ID: Cage)	12.05	3.471
	INEXP-C x Trial day	-2.114	3.264	75.88	-0.648	0.519	Pair ID: Cage	78.12	8.839
	EXP-OTA x Trial day	-7.965	3.137	68.19	-2.539	**0.013**	Cage	5.45E-13	7.38E-07
	INEXP-OTA x Trial day	-6.412	3.073	60.85	-2.087	**0.041**			

Table 4. Summary statistics for linear mixed models for parental care behaviors contrasting treatment x experience. Bird ID, Pair ID, and Cage ID were random effects for all models. Bolded P values are significant (P<0.05).

Behavior	Contrast	Δ Slope	SE	DF	Lower 95% CI	Upper 95% CI	T ratio	P value
Time in Nest	EXP-C vs. EXP-OTA	187.842	80.418	39.57	-21.995	397.679	2.336	0.095
	INEXP-C vs. INEXP-OTA	-28.897	72.536	41.97	-218.169	160.373	-0.398	0.991
	EXP-C vs. INEXP-C	94.327	83.942	39.94	-124.706	313.361	1.124	0.712
	EXP-OTA vs. INEXP-OTA	-122.412	69.145	43.3	-302.835	58.01	-1.77	0.295
Nest guard	EXP-C vs. EXP-OTA	0.977	10.748	24.98	-27.866	29.82	0.091	1
	INEXP-C vs. INEXP-OTA	-26.524	9.647	25.13	-52.412	-0.636	-2.749	**0.042**
	EXP-C vs. INEXP-C	2.745	11.104	23.82	-27.053	32.543	0.247	0.998
	EXP-OTA vs. INEXP-OTA	-24.756	9.232	26.26	-49.53	-0.017	-2.682	**0.049**
Nest maintenace	EXP-C vs. EXP-OTA	0.855	3.481	132.03	-7.936	9.646	0.246	0.998
	INEXP-C vs. INEXP-OTA	4.258	3.161	133.36	-3.725	12.243	1.347	0.548
	EXP-C vs. INEXP-C	-5.037	3.616	116.96	-14.169	4.095	-1.393	0.516
	EXP-OTA vs. INEXP-OTA	-1.633	3.013	133.46	-9.244	5.976	-0.542	0.971

Table 5. 95% Confidence intervals for the difference in slopes (ΔSlope) between the OTA and control groups by experience for the change in behavioral duration (in seconds) vs. trail day (day 0 to day 5), as measured over a 60-minute observation period, with a Tukey-adjustment to the p-values for multiple contrasts.

Response Varial	Model Term	Sum SQ	Mean SQ	DF	F value	P value
Initial Body Mass	Treatment	0.009	0.009	12.651	0.354	0.562
	Experience	0.015	0.015	15.856	0.585	0.455
Max Growth Rate	Treatment	1.629	1.629	15.888	10.986	**0.004**
	Experience	0.438	0.438	16.615	2.9551	0.104
Hatch Rate	Treatment	1.218	1.218	13.581	0.795	0.388
	Experience	0.039	0.039	17.338	0.026	0.873

Table 6. ANOVA table comparing the effects of treatment and experience level for chick effects from LMM with Pair ID nested within Cage ID as a random intercept. Bolded *P*-values are less than 0.05.

Chick Effect	Contrast	Δ Mean	SE	DF	Lower 95% CI	Upper 95% CI	T ratio	P value
Initial Body Mass	Treatment	0.028	0.065	12.651	-0.125	0.181	0.431	0.89
	Experience	0.065	0.067	15.856	-0.093	0.224	0.965	0.566
Max Growth Rate	Treatment	0.793	0.183	15.888	0.364	1.223	4.337	**0.0003**
	Experience	-0.424	0.188	16.615	-0.866	0.018	-2.249	0.062

Table 7. 95% Confidence intervals for the difference in mean (∆Mean) between treatment groups and experience groups for initial body mass (g) and max growth rates (g/day) for all chicks, with a Tukey-adjustment to the p-values for multiple contrasts.

Figures

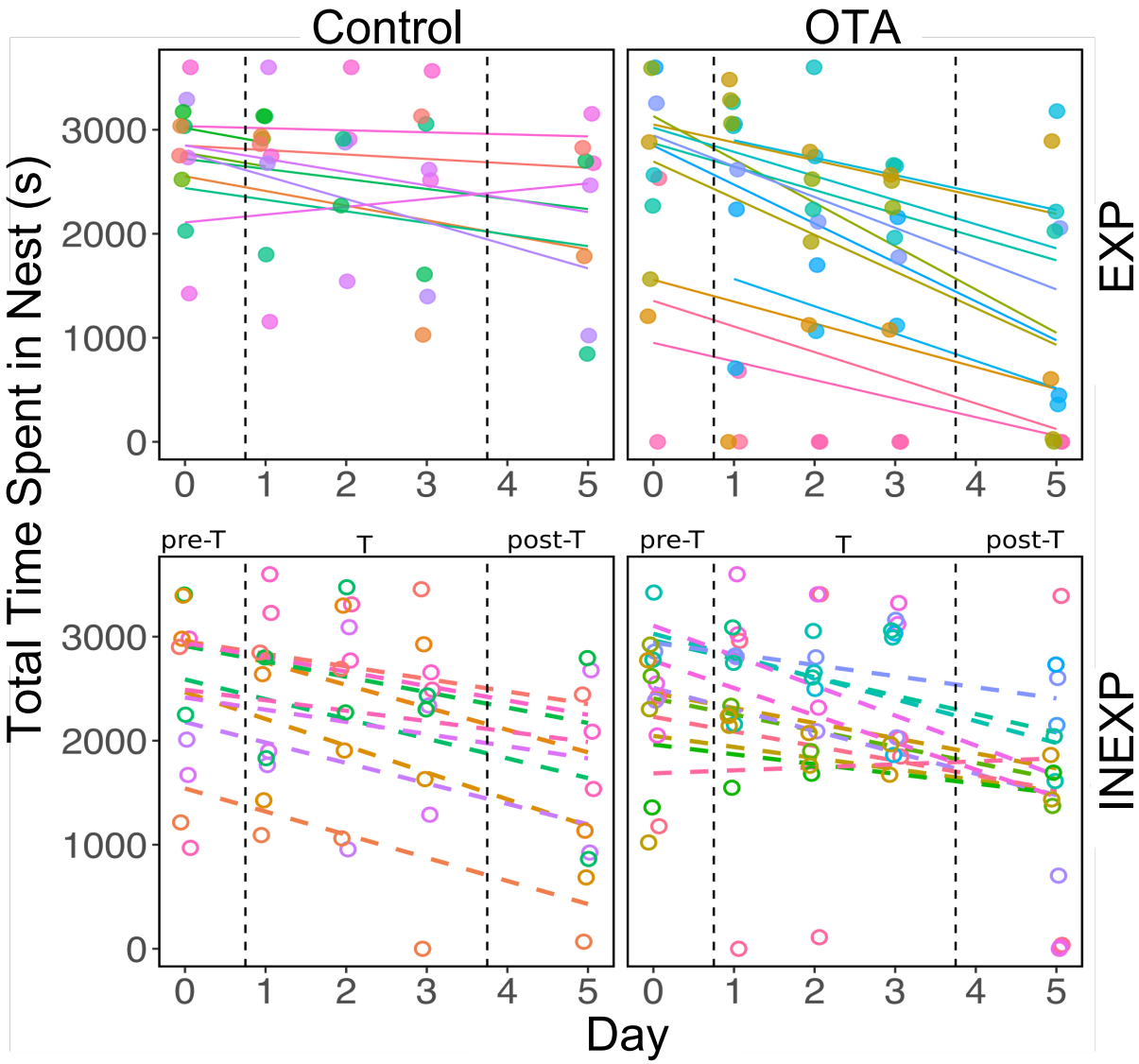

Figure 7. The total time spent in the nest vs. day for all combinations of treatment and experience. Different colored lines indicate random-effect slopes and intercepts fit for each bird. Total time spent in the nest significantly declined across the days for EXP-OTA (P=0.00005), INEXP-OTA (P=0.011), and INEXP-C (P=0.008) birds, but did not significantly change for EXP-C birds (P=0.232). However, post-hoc comparisons between the groups found that they did not significantly differ from each other (all P >0.05)

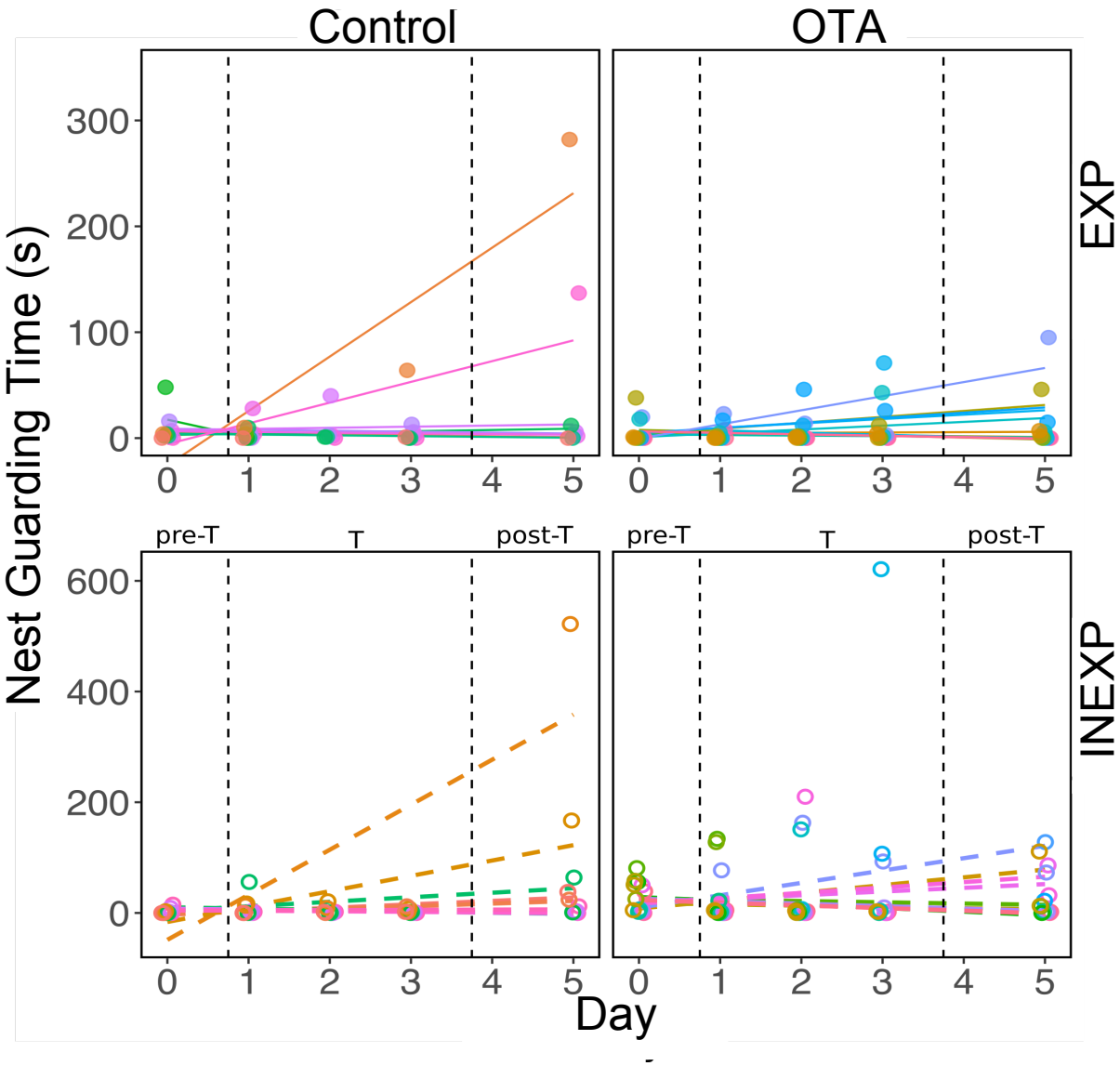

Figure 8. The total time birds spent guarding their nests vs. day for all combinations of treatment and experience. Different colored lines indicate random-effect slopes and intercepts fit for each bird. Total time spent nest guarding significantly increased across the trial days for INEXP-OTA birds (P=0.001) but did not significantly change for EXP-OTA, EXP-C, and INEXP-C birds (all P >0.05). Post-hoc contrasts of slopes were significantly different for INEXP-OTA vs. INEXP-C (P=0.042) and INEXP-OTA vs. EXP-OTA (P=0.049); however, note that the y-axis scale, which was set to include all data points, is approximately twice as large for inexperienced birds compared to the y-axis

scale experienced birds, making it difficult to visualize the greater slope in the INEXP-OTA relative to other treatment x experience groups, particularly when plotting model predictions that include random effects at the scale of individual birds. Post-hoc contrasts of slopes for EXP-C vs. EXP-OTA and EXP-C vs. INEXP-C did not significantly differ from each other (all $P > 0.05$).

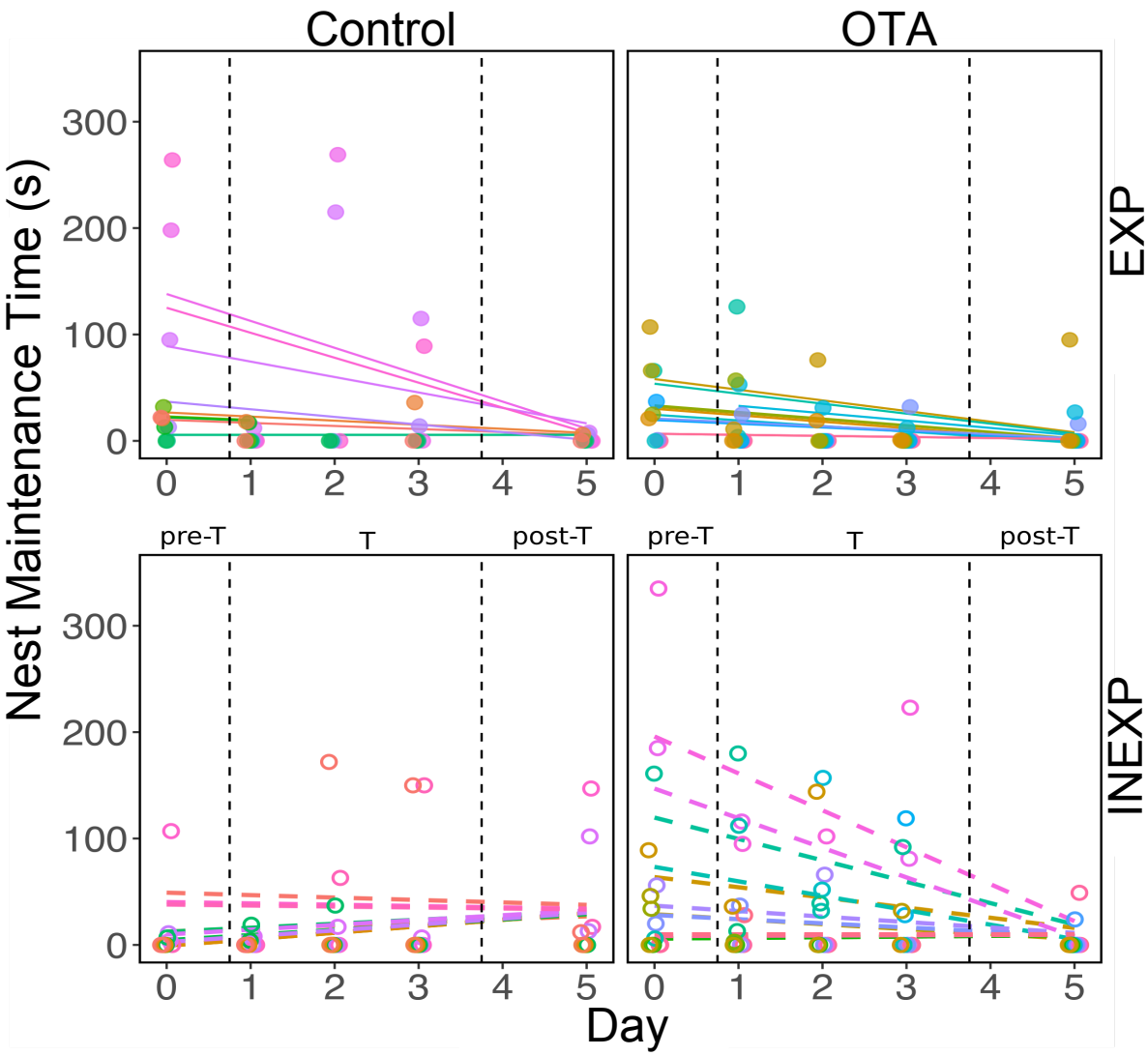

Figure 9. The total time birds spent on nest maintenance vs. day for all combinations of treatment and experience. Different colored lines indicate random-effect slopes and intercepts fit for each bird. Total time spent performing nest maintenance significantly declined across the trial days for EXP-OTA (*P*=0.013), INEXP-OTA (*P*=0.041), and EXP-C (*P*=0.043) birds but did not significantly change for INEXP-C birds (*P*=0.519). However, post-hoc comparisons between the groups found that they did not significantly differ from each other (all *P* >0.05).

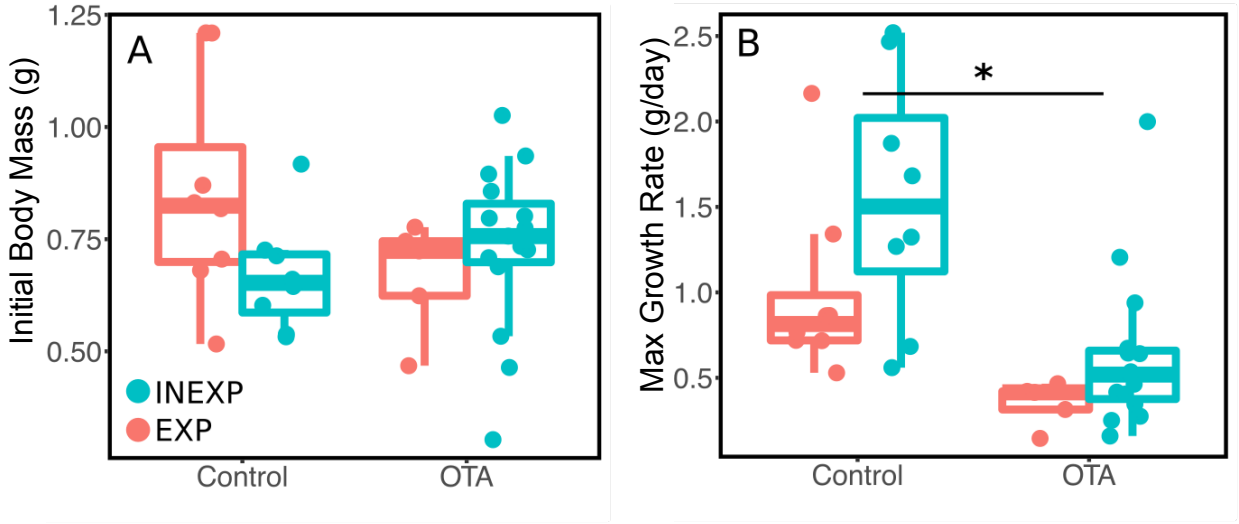

Figure 10. The (A) initial body mass and (B) maximum growth rate of chicks hatched during the experiment, as determined by fitting parameters from the logistic growth function to each individual chick. Chicks with parents that received the OTA had significantly slower maximum growth rates (P=0.004) (B) but did not differ in initial birth weights (A) (P>0.05 for treatment and experience).

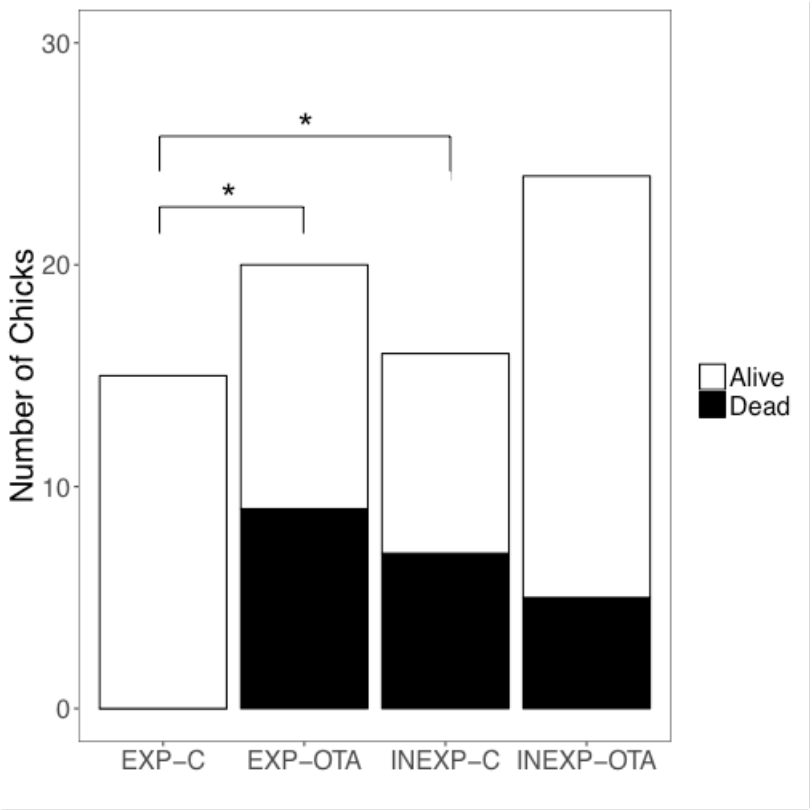

Figure 11. Total number of chicks that lived or died over the course of the experiment. (*) indicates a significant difference in chick mortality between groups. EXP-C had significantly fewer chicks die than EXP-OTA (*P*=0.02) or INEXP-C *(P*=0.02).

CHAPTER FOUR

Committed for the long haul: do nonapeptides regulate long-term pair maintenance in zebra finches?

Abstract: The nonapeptides regulate a number of social behaviors across vertebrates including monogamous pair bonds in mammals. Recent work on zebra finches has shown an important role for these neurohormones in establishing avian pair bonds as well. However, studies on the role of nonapeptides in maintaining pair bonds after pair formation are lacking. The goal of the present study was to investigate the effects of an oxytocin receptor antagonist (OTA) on pair maintenance behaviors in the monogamous zebra finch. I injected established zebra finch pairs over three days with either 5µg of an OTA or a vehicle control, and separated the partners for one hour, after which partners were reunited and their reunion recorded on video for 30 min. Videos were then coded to measure singing, affiliative (allopreening, clumping, following), and aggressive (pecking) behaviors. These behaviors were also measured both on the day before injections to establish a pre-treatment level and two days after the last injection. Control and antagonist treated birds did not differ in the amount of time spent clumping or the frequency of pecking across the experiment. However, both male and female zebra finches that received OTA significantly reduced the amount of time spent following their partner. Females given the OTA treatment reduced allopreening and males given the OTA treatment reduced the frequency of singing bouts directed at their partners relative to controls. These results suggest that the nonapeptides play a role in regulating some, but not all, pair maintenance behaviors in experienced zebra finches.

CHAPTER 4: Nonapeptides and Pair Maintenance in Zebra Finches

Introduction

The nonapeptides oxytocin, vasopressin, and their non-mammalian homologs have been shown to regulate a wide range of social behaviors across vertebrates (Donaldson and Young 2008, Choleris et al. 2013). These results suggest that the neurohormonal mechanisms underlying social behaviors are conserved among distantly related taxa. However, the nonapeptide mechanisms regulating pairing behavior are only well studied in rodents, especially the monogamous prairie vole (*Microtus ochrogaster*) (Young and Wang 2004, Lim and Young 2006). As social monogamy evolved independently multiple times across vertebrates (Whiteman and Côté 2004, Adkins-Regan and Tomaszycki 2007, Lukas and Clutton-Brock 2013), the mechanisms of pair bonding likely differ between prairie voles and other mammals and non-mammals (Goodson and Thompson 2010).

Pair bonds are established in prairie voles after mating occurs and are dependent on oxytocin (OT) for females (Insel and Hulihan 1995, Liu and Wang 2003) and vasopressin (AVP) for males (Liu et al. 2001, Lim and Young 2004). Administration of either an OT receptor antagonist (OTA) for females or an AVP 1a receptor antagonist for males is sufficient to disrupt pair bond formation in naïve voles (females-Liu and Wang 2003, males-Liu et al 2001). However, disrupting nonapeptide action is not sufficient to block pair formation in other species. For example, in the socially monogamous black-tufted marmoset (*Callithrix penicillata*), administration of an OTA did not prevent pair bond formation, though it did significantly reduce partner directed affiliative behavior (Smith et al. 2010). Similarly, monogamous convict cichlids (*Amatitlania nigrofasciata*) treated with a non-specific nonapeptide antagonist delayed

pair bonding, but ultimately did form pairs (Oldfield and Hofmann 2011). Together, these studies support a general role for nonapeptides in pair bonding across vertebrates, but highlight species-specific regulation and the need to study a wide range of taxa to understand the neurohormonal mechanisms underlying monogamy.

The mechanisms regulating pair formation may also differ from those involved in pair maintenance. Dopamine and its receptors (D1 and D2) can either promote or inhibit pair formation in prairie voles, depending on what type of dopamine receptor is active. Activation of D2-like receptors significantly promoted pair formation while activation of D1-like receptors inhibited pair formation in prairie voles (Aragona et al. 2006). By contrast, pair maintenance involved a significant increase in D1-like (but not D2-like) receptors in the nucleus accumbens, leading to selective aggression towards non-partner voles (Aragona et al. 2006). Therefore, the same receptors can play opposing roles during pair formation and maintenance, with D1 inhibiting pair formation but promoting pair maintenance.

Consistent with the experiments on pair bonds in prairie voles, recent work in socially monogamous zebra finches (*Taeniopygia guttata*) found that peripheral administration of an OTA significantly reduced the likelihood of pairing in inexperienced birds (Pedersen and Tomaszycki 2012). Chronic central administration of OTA also significantly disrupted pair formation in inexperienced zebra finches (Klatt and Goodson 2013). However, both studies found that after receiving OTA, some naïve zebra finches still did form pair bonds, suggesting that pair formation may be somewhat independent of nonapeptides. Based on these results and the work in prairie voles, the nonapeptides are likely involved in regulating pair maintenance behaviors in zebra finches (Prior and

Soma 2015).

Recent work in zebra finches also suggests that the nonapeptides may play a different role in initial pair formation than in pair maintenance (Lowrey and Tomaszycki 2014). Zebra finches were allowed to pair naturally and then tested for the abundance of nonapeptide mRNA in the brain at either 48 hours or two weeks after pair formation. In males, courtship behaviors (e.g., directed singing) during the first 48 hours best explained increases in MT (mesotocin, avian homolog of oxytocin) and AVT (arginine vasotocin, avian homolog of vasopressin) in the paraventricular nucleus (PVN) and bed nucleus of the stria terminalis (BSTm) (Lowrey and Tomaszycki 2014). While expression of MT and AVT in the PVN remained high at both 48 hours and two weeks after pairing, expression of AVT in the BSTm was only high after 48 hours and dropped by two weeks. There also was a sex effect of pairing as only clumping (partners perched at rest in direct physical contact) predicted variations in MT mRNA in females and no pair behaviors predicted AVT mRNA in either the PVN or BSTm (Lowrey and Tomaszycki 2014). These results suggest that there may be differences in the mechanisms behind pair bonding and maintenance as well as sex differences in the effects of nonapeptides on pair behaviors. To the best of my knowledge, no study has yet directly examined the effect of the nonapeptides on pair maintenance behaviors in birds.

Although MT and AVT can potentially affect behavior via distinct mechanisms, due to the promiscuity of their receptors it is difficult to disentangle these distinct effects. Like most vertebrates, birds have multiple vasotocin/vasopressin receptors and one OT-like receptor (Baeyens and Cornett 2006). In birds, V1a, VT1, and OT-like VT3

receptors are widespread throughout the brain, particularly in regions associated with social behaviors (Leung et al. 2011). In zebra finches the VT3 receptor has been shown to bind both MT and AVT (Baeyens and Cornett 2006, Leung et al. 2009); therefore, blocking the VT3 receptor with an oxytocin receptor antagonist could inhibit the binding of both MT and AVT. For this reason, this experiment tests whether nonapeptides in general, and not MT or AVT specifically, regulate pair-maintenance behaviors by bonding to the OT-like VT3 receptors in the brain.

I tested whether the zebra finch behaviors involved in maintaining an established pair bond were regulated by nonapeptides MT and AVT by administering an OTA peripherally. I predicted that administration of an oxytocin receptor antagonist could disrupt pair maintenance behaviors in reproductively experienced pairs.

Methods

Subjects and housing

The control and experimental groups each included eight zebra finch pairs (16 males and 16 females across both treatments). All subjects had been bred in the lab. All birds had been freely allowed to choose their partners prior to the start of the study, and all pairs had successfully raised at least one clutch of offspring. Each pair had previously participated in a study on the effects of an oxytocin receptor antagonist (OTA) on parenting. After the parenting experiment, the subjects were kept with their partners in mixed-sex aviaries for at least two weeks to ensure no lasting effects of the previous treatment. The previous treatment groups of the subjects were randomized with respect to the current study. Individual age was also randomized with respect to

treatment.

All subjects were housed in a temperature and humidity controlled room on a 14:10 light:dark schedule for the duration of the experiment. Each individual had four unique leg bands; three colored and one silver ID band with an individualized ID code.

Prior to the start of the study, pairs were randomly selected and assigned to one of four aviaries such that each aviary consisted of four pairs. To control for any cage effects, two pairs from each cage were in the experimental group and the remaining two pairs were in the control group. All aviaries (0.94 m by 0.76 m by 0.94 m) were equipped with seed, grit, cuttlebone and water ad libitum. All aviaries were located within the same room. The Cornell University IACUC approved all methods and procedures of the study.

OTA injections

Pairs were randomly assigned to either the experimental group or a vehicle control group. Both males and females within a pair were given the same treatment. Each bird in the experimental group was given a 0.05 ml intramuscular injection into the pectoral muscle of 5 µg OTA ([d(CH_2)$^1{}_5$, Tyr(Me)^2, Thr^4, Orn^8, des-Gly-$NH^9{}_2$]-Vasotocin trifluoroacetate salt, Bachem) dissolved in 0.9% saline. In rats, this antagonist is 18 times more potent as an OT receptor antagonist than a V1a receptor antagonist (Manning et al. 2008) and has been highly effective in altering rat social behaviors (Neumann et al. 2003, 2006). This antagonist has also effectively disrupted social behaviors in zebra finches (Goodson et al. 2009, Pedersen and Tomaszycki 2012, Klatt and Goodson 2013). 5 ug of this OTA administered through peripheral injection has been previously shown to be effective in disrupting zebra finch pair formation (Pedersen

and Tomaszycki 2012). Control subjects were injected with the same volume of 0.9% saline. Animals were only injected once per day for three days and the effects of each injection likely did not last the entire 24-hour period of each day (Pedersen and Tomaszycki 2012).

Separation and reunion testing

To induce pair maintenance behaviors, I briefly separated each subject from their partner before reuniting them and video recording their behaviors. Birds were separated and then reunited and recorded on each of five days: Pre-treatment (Day 0, no injection), three consecutive days of injections (Days 1-3), and Post-treatment (Day 5, no injection, 48 hours after the third injection). For each recording day, the partners were removed from their home cage and then either immediately placed into two separate small aviaries (Pre-treatment and Post-treatment) or injected (Days 1-3) and placed into two separate small aviaries for 60 minutes. The same two small aviaries were used on each day. The small aviaries for both birds were placed across from their home aviary, and males and females could hear but not see their pair partners. Separation from the partner for as little as one hour induces a glucocorticoid stress response and also leads to an increase in affiliative behaviors upon reunion (Remage-Healey et al. 2003). Though previous studies using this OTA had intervals between injection and behavioral testing ranging from 10-30 min (Samuelsen and Meredith 2011, Pedersen and Tomaszycki 2012), we chose 60 minutes to increase the likelihood of affiliative pair maintenance behaviors (Baran et al. 2016).

After being separated for 60 minutes, both members of a pair were placed in a

new aviary (0.94 m by 0.76 m by 0.94 m) within their homeroom and video recorded for 30 minutes. After recording, the subjects were returned to their home aviary. Videos were scored separately for each male and female by a single trained coder who was blind to treatment. Behaviors scored for pair maintenance (see Table 8) were based on previous studies of pairing behavior in zebra finches (Tomaszycki and Adkins-Regan 2005, Pedersen and Tomaszycki 2012, Baran et al. 2016). Specifically, I scored time spent perched in contact (clumping), allopreening bouts by both birds (allopreen), frequency of song bouts (song bout) and total time spent singing (total song) by males, and following the partner to a new perch within three seconds (follow). I also scored number of pecks (pecking), an aggressive behavior (Table 8).

Data analysis

Data was analyzed using linear mixed-effect models to determine how treatment affected pair maintenance behaviors. All models were run in R (version R 3.4.0) using the “lme” package. I used nested random-effects terms to address both the repeated measurement of individual birds and the potential non-independence of an individual’s behavior due to how their partner behaved or what cage they were in.

For linear mixed effects models of allopreening, follow, and pecking, Bird ID (unique code for each bird) was nested within Pair ID (unique identifier for each pair) and Pair ID was nested within Cage ID (unique code for each cage) as random intercepts and random slopes for the change in behavioral duration or frequency with day. For linear mixed effects models of singing, which is performed only by males (so that only one bird in each Pair ID was repeatedly measured), Bird ID was nested within

Cage ID as random intercepts and random slopes for the change in behavioral duration and frequency with day. For linear mixed effects models of clumping (a single measurement of a behavior exhibit by both birds in a pair) Pair ID was nested within Cage ID as random intercepts and random slopes for the change in behavioral duration or frequency with day.

All models initially included fixed main effects of sex and treatment and 2- and 3-way interactions between sex by treatment and sex by treatment by day. I then removed insignificant fixed effects in a backwards, stepwise manner until only significant terms remained. Treatment and day were treated as fixed effects for all models. Control and OTA groups were considered significantly different from each other if there was a significant treatment by day interaction, as determined by analysis of variance (ANOVA) (Table 9).

For allopreening, the behavior with a significant 3-way sex x treatment x day interaction, a post-hoc Tukey's Test was used to determine if significant differences exist in the slopes of the change in the behavioral duration vs. day between sexes in the same treatment (2 contrasts) and between treatments of the same sex (2 contrasts, for a total of 4). In the absence of significant sex main- or interaction-effects in the ANOVA of the linear mixed effect models, the reported F-tables are for behaviors measured in both males and females (except for singing, which is performed only by males) (Table 9).

Following Kline (2004), non-standardized effect sizes in their original units are reported, rather than approximations of effect size statistics such as Cohen's *d*, due to the difficultly of estimating the pooled variance of groups of semi-independent

measurements in models with complicated random-effect structures (*see also* Nakagawa and Cuthill 2007). Thus, the effect size is a measurement of the difference in slopes of the change in the behavioral duration or frequency vs. day. For example, for a contrast of behavioral duration between the control and OTA treatments, an effect size of 100 (seconds/day) means that, on average, the birds in the control treatment increased the behavior by an additional 100 seconds/day relative birds in the OTA treatment.

In order to assess the sign, magnitude, and uncertainty around the treatment effects, 95% confidence intervals were generated using the "lsmeans" packaged in R for the difference in slopes between the OTA and control treatment for the change in duration or frequency of a behavior vs. day (Table 10). The reported estimates indicate both the magnitude and sign of treatment effects (positive value, behavior increased in OTA vs. control; negative value, behavior decreasing in OTA vs. control). The associated p-values give the significance of each slope contrast; in general, when the 95% confidence interval does not overlap with zero, the contrast is significant at $p<0.05$. For allopreening bouts, 95% confidence intervals were constructed around the difference in slopes of the change in the behavioral duration vs. day between sexes in the same treatment (2 contrasts) and between treatments of the same sex (2 contrasts), with a Tukey-correction for 4-contrasts to both the reported p-values and the width of the reported confidence intervals.

Results

Pair Behaviors

Clumping: Control pairs and OTA pairs did not significantly differ in the amount of time spent clumping over the course of the experiment ($F_{13.922}$=0.401, P=0.536) Fig. 12AB).

Individual Behaviors

Allopreening: For allopreening bouts, there was a significant three-way interaction of treatment by sex by day ($F_{43.641}$=4.537, P=0.039). Allopreening bouts for OTA females did not change across the experiment whereas control females significantly increased the number of allopreening bouts performed across the experiment [Difference in Slope-DSlope: -0.904 ± 0.343 bouts/day (difference ± SE for this and all other reported effect sizes), $T_{20.77}$=-2.589, P=0.05] (Fig. 13CD). Control females also significantly increased the number of allopreening bouts they performed compared to control males (DSlope: 0.931 ± 0.294 bouts/day, $T_{14.14}$=3.163, P=0.011) (Fig. 13AC). Male OTA and control birds did not significantly differ from one another (DSlope: -0.02 ± 0.343 bouts/day, $T_{20.77}$=-0.058, P=1) (Fig. 2AB). Female and male OTA birds also did not significantly differ from one another (DSlope: 0.047 ± 0.293 bouts/day, $T_{13.85}$=0.162, P=1) (Fig. 13BD).

Follow: There was a significant day by treatment interaction for follow bouts ($F_{47.605}$=5.47, P=0.023), with OTA birds reducing follow bouts relative to control birds (DSlope: -0.381 ± 0.163 bouts/day) (Fig. 14AB).

Pecking: Pecking bouts for control birds and OTA birds did not significantly differ over the course of the experiment ($F_{14.084}$=0.679, P=0.423) (Fig. 15AB).

Male singing

Song bouts: There was a significant day by treatment interaction for song bouts

($F_{19.218}$=4.428, P=0.048), with the number of song bouts performed by OTA declining relative to control males (DSlope: -1.111 ± 0.528 bouts/day, $T_{19.22}$=-2.104, P=0.048) (Fig. 16AB).

Total song: Total time spent singing across the experiment for control and OTA males was not significantly different ($F_{24.889}$= 2.986, P=0.096) (Fig. 17AB).

Confidence Intervals

For allopreening bouts, the difference in slopes between OTA and control females was estimated -0.904 (95% confidence interval, CI, range: -1.815 to -0.007 bouts/day) and the difference in slopes between control females and males was 0.931 (95% CI: 0.151 to 1.712). For following bouts, the difference in slopes is estimated as -0.381 (95% CI range: -0.709 to -0.053 bouts/day). For song bouts the difference in slopes is estimated as -1.111 (95% CI range: -2.215 to -0.007 bouts/day). The differences in slopes and 95% CI for non-significant results (clumping, pecking, total song) are found in Table 10.

Discussion

I tested whether nonapeptides regulate pair maintenance by administering a general OTA to established zebra finch pairs and measuring its effects on pair maintenance behaviors. Administration of OTA significantly affected some pair maintenance behaviors (following for both sexes, allopreening for females, song bouts for males), but had no effect on others (clumping and pecking for both sexes). These results suggest that the nonapeptides and the OT-like VT3 receptor, which have been shown to regulate pair formation in zebra finches, continue to regulate some pair-

maintenance behaviors in long-term zebra finch pairs.

I found that OTA significantly reduced the number of times birds followed their partners. These results suggest that OTA may reduce an individual bird's motivation to be near their partner, causing them to follow their partner to fewer perches. Similarly, unpaired female zebra finches receiving OTA have been shown to be less motivated to pair (Pedersen and Tomaszycki 2012). This lack of motivation may be more general than pair maintenance behaviors and may instead be a symptom of decreased gregariousness. Knockdown of MT in the paraventricular nucleus of the hypothalamus (PVN) of zebra finches reduced side-by-side perching and vasotocin (AVT) knockdown in the PVN significantly reduced gregariousness in both sexes (Kelly and Goodson 2014). Though I administered an oxytocin receptor antagonist, the OT-like VT3 receptor may bind both MT and AVT (Leung et al. 2009), such that the observed reduction in following may be due to blocking MT, AVT, or both. Therefore, in zebra finches, MT and AVT may work in tandem in the PVN to regulate gregariousness.

Decreased gregariousness may also be responsible for the effect of OTA on female allopreening. Though control and OTA males did not significantly differ in the amount of allopreening they performed, control females performed significantly more allopreening than both OTA females and control males. These results support one study on pair formation in zebra finches, where ICV infusions of OTA significantly reduced allopreening in females but not males (Klatt and Goodson 2013). By contrast, our results are inconsistent with another study that found peripheral injections of OTA during pair formation had no effect on allopreening in zebra finches, although variability was high within the groups (Pedersen and Tomaszycki 2012). These differences may

be methodological, as this study injected birds over three days and the Pedersen and Tomaszycki (2012) study only injected birds over two days. Therefore, nonapeptide regulation of allopreening during pair formation and maintenance in zebra finches may be sex-specific.

Other studies of social behaviors in zebra finches have found sex specific effects of nonapeptides. Peripheral administration of OTA significantly reduced the amount of time females but not males spent in close proximity with familiar cage mates (Goodson et al. 2009). Knockdown of MT in the PVN also reduced gregariousness in females but not males (Kelly and Goodson 2014). Similarly, sex-specific regulation of social behaviors by nonapeptides has been found in a number of mammals. Sex-specific roles for nonapeptides are found in prairie voles, where OTA disrupts pairing in females (Liu and Wang 2003) and vasopressin receptor antagonists disrupt pairing in males (Liu et al 2001). Arginine vasopressin (mammalian homolog of AVT) has also been shown to influence affiliative and antisocial behaviors in humans in a sex-specific manner (Thompson et al. 2006). Thus, the observed sex-specific effects of OTA on allopreening are consistent with a range of studies suggesting that nonapeptides regulate social behaviors in a sex-dependent manner.

Though I found OTA to significantly allopreening and following, the size of the effect, as indicated by the (unstandarized) difference in the slopes describing the how behavioral duration or frequency changed over the 5 days of observation, were relatively small for both behaviors. Although the changes in these behaviors between treatment groups were statistically significant, it remains unclear if effects of this magnitude are sufficient to disrupt established ZF pairs. Future studies should focus on

increasing study length or sample size to determine if these significant results are also biologically significant.

OTA also negatively affected singing, as OTA males significantly reduced the number of song bouts they performed across the experiment, whereas the number of song bouts performed by control males remained relatively constant. These results support previous work showing that, during pair formation in zebra finches, OTA males decreased singing (Pedersen and Tomaszycki, 2012), and that males treated with an arginine vasotocin receptor antagonist (V1aR) as juveniles sang significantly less as adults than controls during a pair reunion trial (Baran et al. 2016). However, when zebra finch males received chronic intracerebroventricular (ICV) infusions of OTA, they increased song production compared to controls (Klatt and Goodson 2013). Methodological differences in the studies could account for the different outcomes in male song, as this study and Pedersen and Tomaszycki (2012) both found similar results with respect to song using a peripheral injection of OTA, whereas Klatt and Goodson (2013) found the opposite result using ICV administered OTA. Regardless of whether OTA significantly decreased or increased singing, song was affected across all of these studies, supporting the hypothesis that nonapeptides regulate song in male zebra finches.

Though I found OTA to significantly affect some pair maintenance behaviors, pecking and clumping were unaffected by the antagonist. As there was no trend for either behavior across the treatment days, these behaviors may be outside of nonapeptide regulation after pair bonds form. Aggression was also unaffected by OTA in similar studies of pair formation in zebra finches (Pedersen and Tomaszycki 2012,

Klatt and Goodson 2013). For example, in one experimental manipulation of pair formation, zebra finch females reduced clumping after the first day after peripheral injection of OTA, though the same effect was not observed in males and clumping on subsequent days was unaffected by the antagonist for either sex (Pedersen and Tomaszycki 2012). Therefore, the nonapeptides may regulate clumping during pair formation (Pedersen and Tomaszycki 2012) but not pair maintenance. Clumping and pecking may also be under regulation by the nonapeptides but through a different receptor (such as V1a or VT1), as the OTA used in this study is specific to the OT-like VT3 receptor (Manning et al. 2008).

Though I found no effect of OTA on clumping or pecking, the OTA may be effective at higher or lower doses as nonapeptide regulation of behavior tends to be dose-dependent. In rats, low but not high doses of OT facilitate social recognition (Popik et al. 1992, Benelli et al. 1995). When pairing OT with an OT receptor antagonist (OTA), low doses of OT and OTA eliminated any positive OT effects on social recognition, but high doses of OT and OTA had a positive effect on social recognition (Benelli et al. 1995). In female prairie voles, administration of OT had dose-dependent effects on pair bonding, with high but not low doses reducing female partner preference and likelihood to form pair bonds (Bales et al. 2007).

Pair formation behaviors in zebra finches were also differentially affected depending on the size of the OTA dose. In females, the medium (5μg) but not low (1μg) or high (10μg) OTA dose significantly reduced the time individuals spent in the nest-box with males (Pedersen and Tomaszycki 2012). In males, the low and medium but not high doses significantly reduced song on the first day, though all doses reduced song

on subsequent days. Likelihood to pair was also dose-dependent in males, as males treated with the medium but not low or high dose of OTA were significantly more likely to remain unpaired compared with control males (Pedersen and Tomaszycki 2012). However, regardless of the dose, there was no effect of the OTA on aggression for females or males and clumping for males (Pedersen and Tomaszycki 2012). For females, clumping was negatively affected by all doses on the first day but none of the doses had any effect on clumping on the subsequent days of the experiment. Therefore, though possible that a larger or smaller dose of OTA could affect clumping and aggression, it seems more likely that these behaviors are outside of nonapeptide regulation through the OT-like VT3 receptor during pair maintenance.

Conclusions

This study demonstrated the importance of the nonapeptides for some, but not all, pair maintenance behaviors in long-term pairs. Relative to control birds, the OTA treated male zebra finches sang less to their partners, female zebra finches spent less time allopreening their partners, and both sexes performed fewer following bouts. However, neither clumping nor pecking were affected by the OTA, indicating these behaviors may be independent of nonapeptides during pair maintenance. These results, when contrasted with previous studies on nonapeptide regulation during pair bonding, illustrate how the regulation of different pair behaviors can either change or be conserved within an individual depending on life history stage. Overall, this research supports the hypothesis that nonapeptides, which are known to regulate a suite of

behaviors involved in pair-formation, continue to play an important role in regulating affiliative behaviors during pair maintenance.

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Tables

Behavior	Female or Male	Duration or Frequency	Description
Clumping	Both	Duration	Perched at rest in direct physical contact
Allopreening	Both	Frequency	Grooming of partner
Song bouts	Male	Frequency	Number of song bouts directed at partner
Total Song	Male	Duration	Total time spent singing directed at partner
Follow	Both	Frequency	Follow partner within three seconds to new perch
Pecking	Both	Frequency	Sharp beak jab at partner

Table 8. Pair maintenance and aggressive behavior coded for zebra finches. Pecking is aggressive.

Behavior	Fixed Effect	Sum SQ	Mean SQ	DF Num	DF Den	F value	P value
Clumping	Treatment	4646	4646	1	9.401	0.037	0.851
	Trial day	771241	771240	1	13.847	6.169	**0.026**
	Treatment x Trial day	50101	50101	1	13.922	0.401	0.536
Allopreen	Treatment	1.571	1.571	1	12.49	0.37	0.554
	Trial day	1.485	1.485	1	6.283	0.349	0.574
	Sex	0.935	0.935	1	38.668	0.22	0.641
	Treatment x Trial day	12.098	12.098	1	13.89	2.849	0.113
	Treatment x Sex	0.02	0.02	1	38.668	0.005	0.945
	Trial day x Sex	23.609	23.609	1	43.641	5.56	**0.023**
	Treatment x Trial day x Sex	19.264	19.264	1	43.641	4.537	**0.039**
Follow	Treatment	5.9977	5.9977	1	12.598	2.353	0.149
	Trial day	9.471	9.471	1	37.019	3.716	0.061
	Treatment x Trial day	13.9404	13.9404	1	47.605	5.47	**0.023**
Pecking	Treatment	1.863	1.863	1	14.596	4.547	**0.05**
	Trial day	0.132	0.132	1	14.084	0.322	0.578
	Treatment x Trial day	0.385	0.385	1	14.084	0.94	0.348
Song Bout	Treatment	6.963	6.963	1	14.229	0.679	0.423
	Trial day	56.549	56.549	1	19.218	5.521	**0.029**
	Treatment x Trial day	45.355	45.355	1	19.218	4.428	**0.048**
Total Song	Treatment	68.37	68.37	1	14.351	0.244	0.629
	Trial day	1593	1593	1	24.889	5.68	**0.025**
	Treatment x Trial day	837.48	837.48	1	24.889	2.986	0.096

Table 9. ANOVA statistics for linear mixed models for all behaviors. Bird ID, Pair ID, and Cage ID were random effects for all models. Bolded P values are significant ($P<0.05$).

Behavior	Contrast	Δ Slope	SE	DF	Lower 95% CI	Upper 95% CI	T ratio	P value
Clumping	OTA vs Control	-31.233	49.34	13.92	-137.104	74.639	-0.633	0.537
Allopreen	OTA vs Control females	-0.904	0.343	29.36	-1.815	-0.007	-2.632	**0.05**
	OTA vs Control males	-0.02	0.343	29.36	-0.931	0.891	-0.059	1
	OTA females vs males	0.047	0.293	43.25	-0.729	0.823	0.162	1
	Control females vs males	0.931	0.294	44.03	0.151	1.712	3.165	**0.011**
Follow	OTA vs Control	-0.381	0.163	47.61	-0.708	-0.053	-2.339	**0.024**
Pecking	OTA vs Control	-0.084	0.087	14.08	-0.27	0.102	-0.97	0.348
Song Bout	OTA vs Control	-1.111	0.528	19.22	-2.215	-0.007	-2.104	**0.048**
Total Song	OTA vs Control	-4.311	2.495	19.62	-9.45	0.828	-1.728	0.096

Table 10. 95% Confidence intervals for the difference in slopes (Δ Slope) between the OTA and control treatments for the change in behavioral duration or frequency vs trail day. For Allopreening, differences are reported between the slopes of different treatment groups within a sex (e.g., OTA females vs Control females) and for different sexes within a treatment group (Control females vs. males), with a Tukey-adjustment to the p-values for multiple contrasts.

Figures

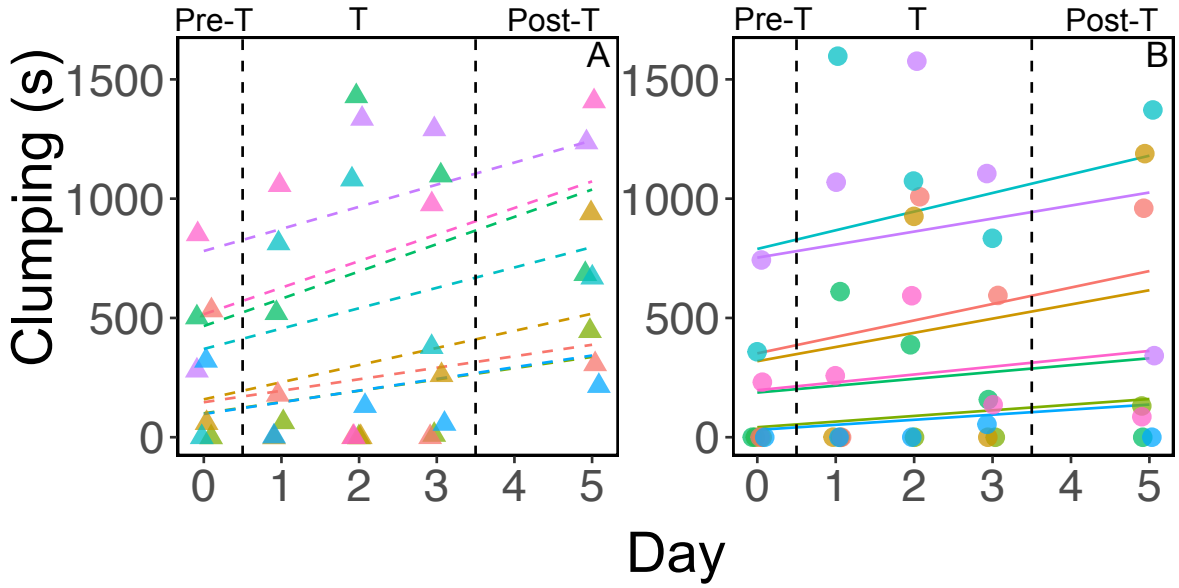

Figure 12AB. Total time spent clumping on each day for control pairs (A) and oxytocin receptor antagonist (OTA) pairs (B). Dotted lines delineate treatment (T) Days 1-3. Day 0 is the Pre-Treatment (Pre-T) day and Day 5 is the Post-Treatment (Post-T) day. The change in time spent clumping over the course of the experiment did not significantly differ between control (A) and OTA pairs (B) (F=0.401, P=0.536).

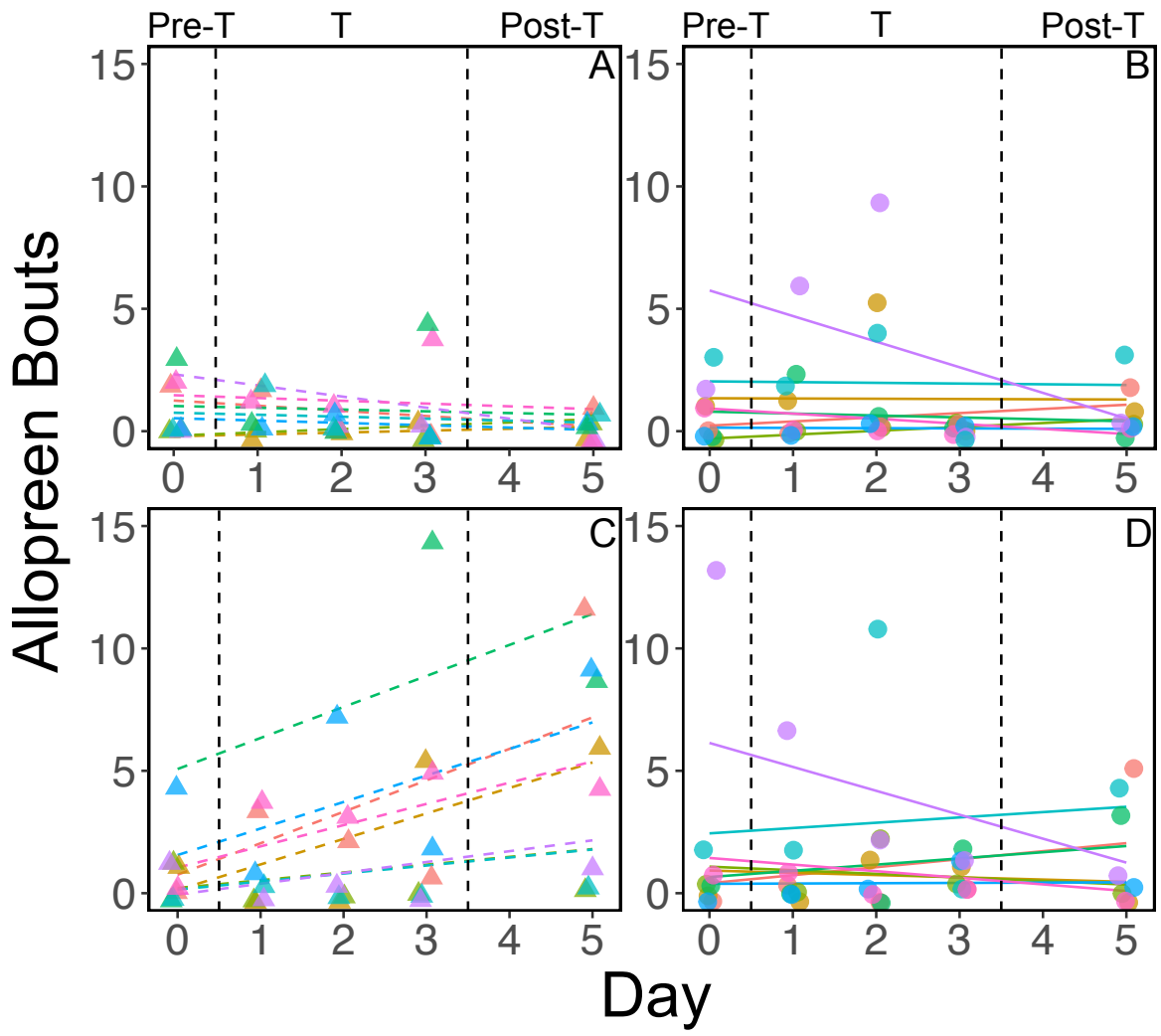

Figure 13A-D. Total number of allopreening bouts performed across the experiment for control males (A), oxytocin receptor antagonist (OTA) males (B), control females (C), and OTA females (D). Dotted lines delineate treatment (T) Days 1-3. Day 0 is the Pre-Treatment (Pre-T) day and Day 5 is the Post-Treatment (Post-T) day. There was a significant treatment by day by sex interaction (P=0.039). This interaction was driven by a significant difference between control and OTA females (P=0.05) (CD) and between control females and control males (P=0.011) (AC). Male control and OTA birds did not significantly differ from one another (P=1) (AB). Female and male OTA birds also did not significantly differ from one another (P=1) (BD).

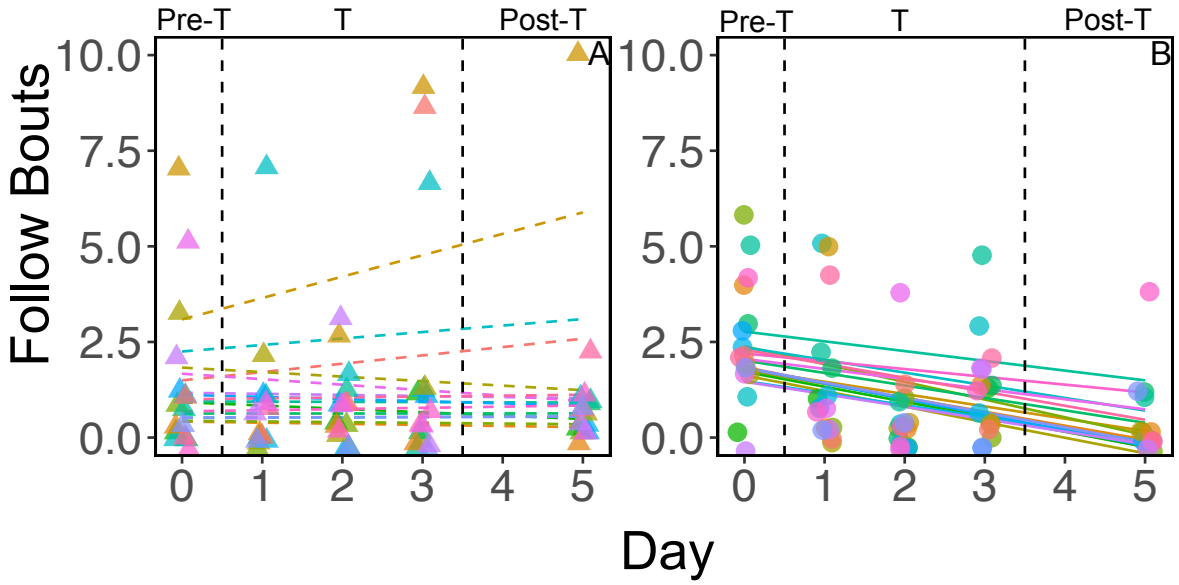

Figure 14AB. Total number of follow bouts performed on each day for control individuals (A) and oxytocin receptor antagonist (OTA) individuals (B). Dotted lines delineate treatment (T) Days 1-3. Day 0 is the Pre-Treatment (Pre-T) day and Day 5 is the Post-Treatment (Post-T) day. Control and OTA follow bouts across the experiment significantly differed from each other (P=0.023).

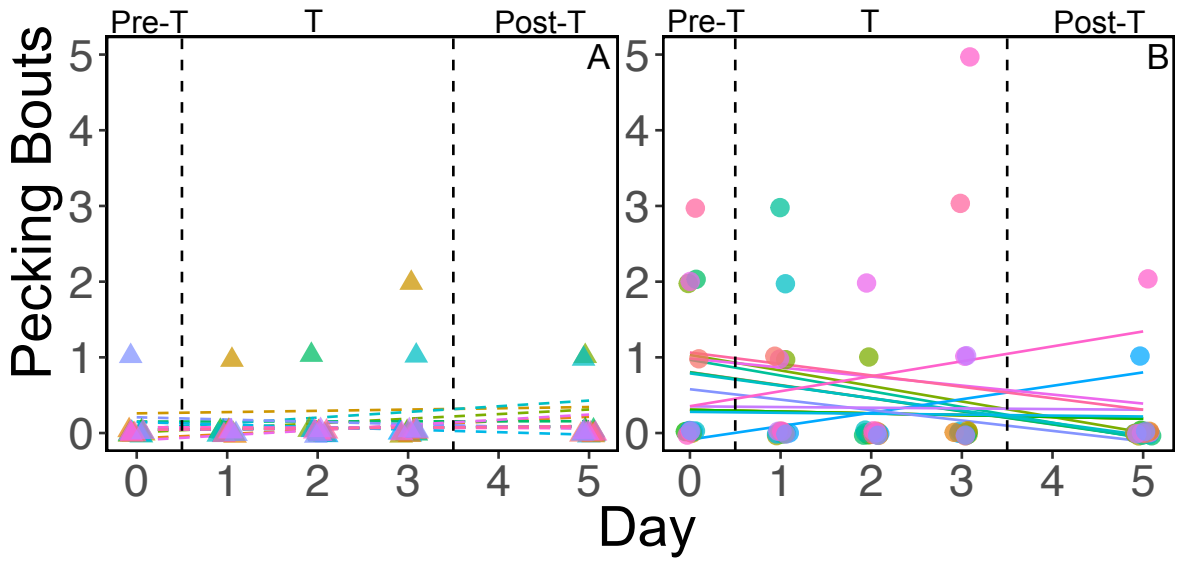

Figure 15AB. Total number of pecking bouts performed across the experiment for control (A) and oxytocin receptor antagonist (OTA) (B) birds. Dotted lines delineate treatment (T) Days 1-3. Day 0 is the Pre-Treatment (Pre-T) day and Day 5 is the Post-Treatment (Post-T) day. OTA and control birds did not significantly differ from each other when comparing pecking bouts across the days (P=0.348).

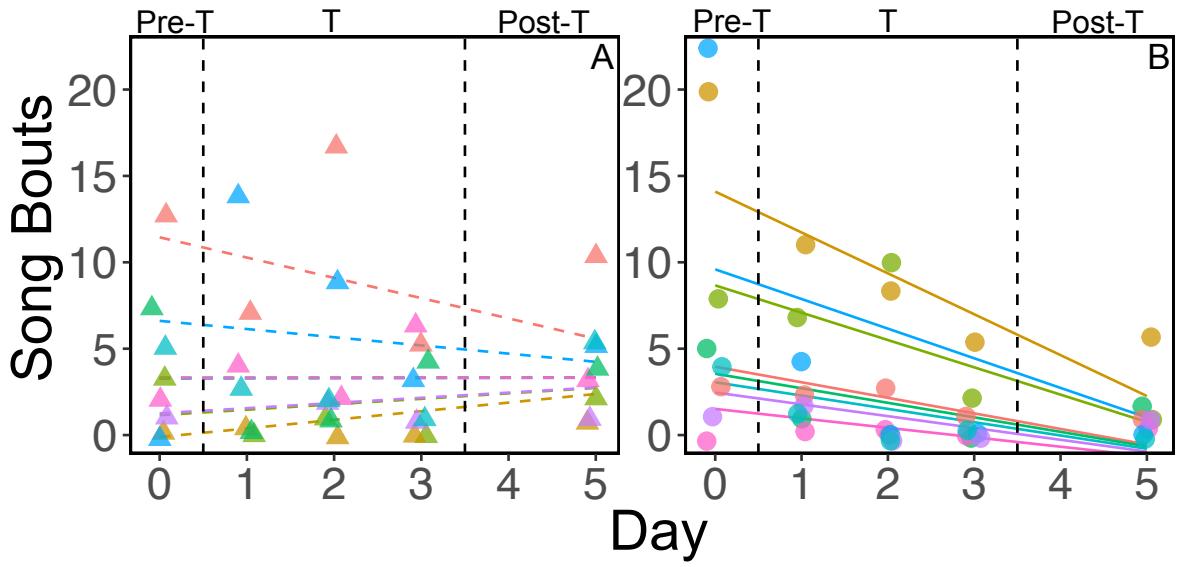

Figure 16AB. Total number of song bouts performed across the experiment for control (A) and oxytocin receptor antagonist (OTA) (B) males. Dotted lines delineate treatment (T) Days 1-3. Day 0 is the Pre-Treatment (Pre-T) day and Day 5 is the Post-Treatment (Post-T) day. Number of song bouts performed by OTA but not control males significantly declined across the experiment (P=0.048).

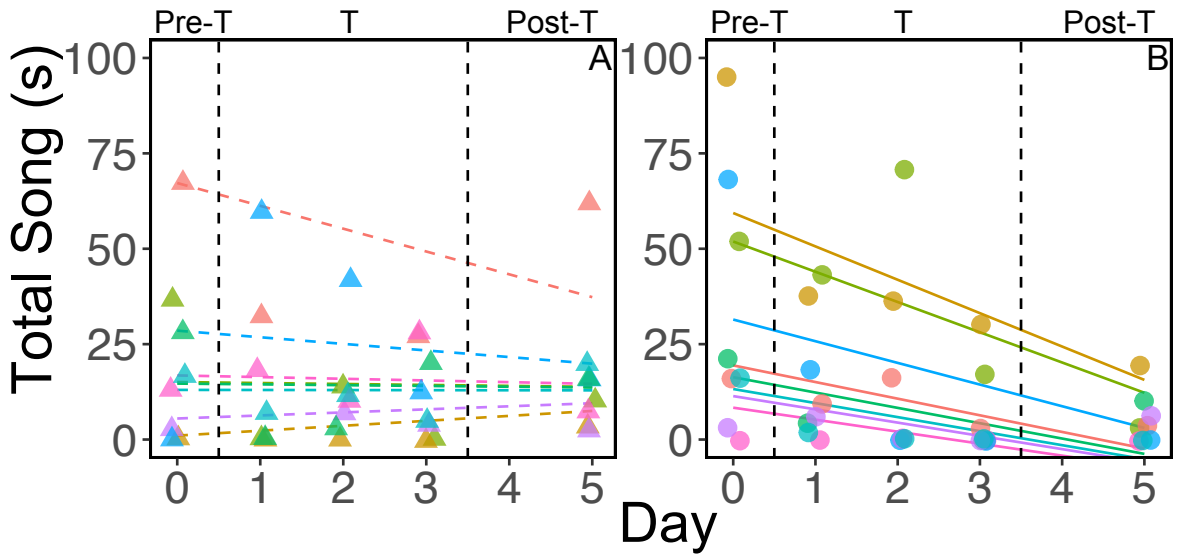

Figure 17AB. Total time spent singing across the experiment for control (A) and oxytocin receptor antagonist (OTA) (B) males. Dotted lines delineate treatment (T) Days 1-3. Day 0 is the Pre-Treatment (Pre-T) day and Day 5 is the Post-Treatment (Post-T) day. Control and OTA males did not significantly differ from one another (*P*=0.096).

CHAPTER FIVE

Conclusions

Introduction

The goal of my dissertation was to understand why individuals in the same environment varied in their expression of social behaviors, in particular, how differences in the underlying hormones within an individual could lead to the expression of different behaviors. While evolutionary theory has described numerous conditions under which social behavior should evolve (Axelrod and Hamilton 1981, Nowak and Sigmund 1993, McNamara et al. 2008), these theories treat behavioral traits as sets of costs and benefits, where selection can structure populations according to the most expedient strategy. This approach has identified environmental conditions that pull individual and group fitness into alignment, but has treated the mechanisms that underlie complex behaviors as a black box. In contrast, studies on the proximate mechanisms inside the body provide a constrained perspective on the traits that selection can act upon. This mechanistic approach does not merely illuminate the contents of the black box, but can alter the qualitative predictions of evolutionary models when the two are integrated together (van den Berg and Weissing 2015).

Social behaviors, particularly pair bonding and parental and cooperative care, serve important ecological functions and are often vital for offspring survival (Clutton-Brock 1991). Though there are a number of theories on when and why pair bonds (Reichard and Boesch 2003), parental care (Clutton-Brock 1991) and cooperative breeding (Koenig and Dickinson 2004) should evolve, they do not specify the mechanism by which they evolve (Koenig and Dickinson 2004, Rhiel 2013). As all social

behaviors have proximate causes, including individual hormonal state, previous social experience, and current conspecific social behavior, research that aims to understand both proximate and evolutionary forces shaping behavior can build a holistic understanding of both how and why social behavior evolves.

Methods and Results

My dissertation research focused on understanding the physiological mechanisms underpinning complex social behavior in birds. I measured and/or manipulated social- and parental-care hormones in active cooperative breeders, paired birds, and parenting birds in both the lab and the wild**.** I found that concentrations of corticosterone did not differ between helper and breeder Mexican jays (*Aphelocoma wollweberi*) or between Mexican jays and Woodhouse's scrub jays (*A. woodhouseii*), though time of year was correlated with corticosterone concentrations in both species (Chapter two). I also determined that administration of an oxytocin receptor antagonist negatively affected parental care outcomes (Chapter three) and some but not all pair maintenance behaviors in zebra finches (*Taeniopygia guttata*) (Chapter four).

In Chapter two, I discussed how differences in life stage did not affect plasma corticosterone concentrations in Mexican jay breeders and helpers. The glucocorticoid reproduction inhibition hypothesis predicts that breeding behaviors in helper individuals are physiologically suppressed by elevated glucocorticoid (a group of hormones including corticosterone) concentrations compared with breeders (Saltzman et al. 1998, Young et al. 2006). In Mexican jays, the lack of difference in corticosterone concentrations between helpers and breeders and between adolescents and adults may

reflect the relatively stable flock dominance hierarchy. As other cooperative species with stable group relationships also do not exhibit differences in concentrations of corticosterone between helpers and breeders (Florida scrub jay, Schoech et al. 1997; white-browed sparrow weavers, Wingfield et al. 1991; Harris's hawks, Mays et al. 1991), group stability or lack there of may be the key driver of differences in corticosterone concentrations between breeders and helpers instead of reproductive inhibition.

More generally, these results support previous work on group living animals, where group instability leads to the highest concentrations of corticosterone throughout all members (Sapolsky 2005). Therefore, I propose that group stability actually determines whether helpers in a cooperatively breeding species experience reproduction inhibition. This addition to the glucocorticoid reproduction inhibition hypothesis further explains the disparate results as the species with support for the hypothesis (Young et al. 2006) likely have unstable group structures, while those species with no support for the hypothesis (Blumstein et al. 2016) may have more stable groups.

In the jays, corticosterone concentrations did not differ between Mexican jays and Woodhouse's scrub jays. However, for both species, corticosterone significantly declined throughout the breeding season, with highest concentrations occurring in March and lowest concentrations during May and June. These results support the glucocorticoid seasonal plasticity hypothesis, which proposes that seasonal changes in glucocorticoid concentrations are related to a species' investment in current vs. future reproduction (Casagrande et al. 2018). Species that invest relatively more in current reproduction are predicted to have higher glucocorticoid concentrations during the

breeding season than the non-breeding season (Bokony et al. 2009, Hau et al. 2010), whereas species investing more in future reproduction will have lower or unchanged concentrations during the breeding season (Casagrande et al. 2018). As both Mexican jays and Woodhouse's scrub jays lay small clutches (Carmen 1989, Brown and Brown 1990), their lack of increase in glucocorticoid concentrations during the breeding season is consistent with both species investing more in future vs. current reproduction.

In Chapter three, I found that an oxytocin receptor antagonist and parental experience both significantly effected nest guarding in zebra finches, with nest guarding increasing in inexperienced birds receiving the antagonist relative to experienced antagonist birds and inexperienced control birds. However, neither the antagonist nor experience significantly effected time spent in the nest or performing nest maintenance across the experiment. However, there was a significant effect of the antagonist on chick outcomes. Chicks reared by parents that received the OTA had significantly lower growth rates than chicks reared by control parents. Chicks in the inexperienced control and experienced antagonist groups also had significantly higher mortality than chicks in the experienced control group. These results support the hypothesis that the nonapeptides are involved in some parental care behaviors.

I found similar results when studying the role nonapeptides play in regulating long-term pair bonds in zebra finches in Chapter four. While nonapeptides have previously been shown to disrupt the formation of pair bonds in inexperienced zebra finches (Pedersen and Tomaszycki 2012), in Chapter four I observed this effect in established pairs. I found that an oxytocin receptor antagonist negatively affected some (following in both sexes, female allopreening, male singing) but not all (clumping) pair

maintenance behaviors. These results support the hypothesis that certain pair maintenance behaviors remain under hormonal regulation throughout the relationship, while other behaviors may become more hormonally independent after pair formation.

Conclusions

Overall, this research sheds light on a number of complex biological processes including the evolution and hormonal basis of social behaviors. These results support and continue the work investigating the hormonal basis of cooperative breeding, parental care, and pair maintenance in birds while providing novel insights into how experience, social environment, and life stage interact with hormonal mechanisms to regulate behavior.

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APPENDIX

Counting on your friends: the role of social environment on quantity discrimination*

Behavioral Processes 128: 9-16.

Abstract: Quantity discrimination has been established in a range of species. However, most demonstrations of quantity discrimination control for social factors by testing animals individually. I tested whether sociality affects quantity discrimination in the wild by comparing the performances of the highly social Mexican jay (MJ; *Aphelocoma wollweberi*) and the territorial Western scrub jay (WJ; *A. californica*). The birds were given a choice between two lines of peanuts that differed in initial quantity ranging from 2 vs 8 to 14 vs 16. Their choices were recorded until all peanuts were eaten or cached. Whereas non-social WJ selected the larger quantity across all the trials significantly more than chance, social MJ selected the larger line only when the difference in the number of peanuts between lines was small. In MJ, individual choice when selecting the large or small quantity was influenced by what line the previous bird had chosen when the difference in lines was large, with followers significantly more likely to select the smaller quantity. WJ were not significantly affected by the choices of other individuals. The only factors that influenced WJ choice were ratio and total differences between the two quantities. These results suggest that in certain scenarios, both species can discriminate between different quantities. However, MJ were greatly influenced by social factors, a previously untested factor, while WJ were only influenced by ratio and total difference between the quantities, consistent with findings in other species. Overall, this

study demonstrates the important role of sociality in numerical cognitive performance, a previously overlooked factor.

APPENDIX: Quantity Discrimination in *Aphelocoma* Jays

Introduction

Whether animals have true numerical sense is a topic both intensely studied (Shettleworth 2010; Stancher 2013; Cronin 2014; Gabor and Gerken 2014; Rugani et al. 2014; Ujfalussy et al. 2014) and debated (Cordes and Brannon 2009; Cantlon et al. 2010; Agrillo et al. 2011). The majority of animals investigated so far show some ability to distinguish between discrete quantities, with the bulk of these studies focusing on small comparisons in the lab (i.e. 1 vs 4, 2 vs 5) (Farnsworth and Smolinski 2006; Beran 2012; Gabor and Gerken 2014), though more recent work on larger comparisons (i.e. 6 vs 8, 8 vs 16) (Garland et al. 2012; Bogale et al. 2014; Rugani et al. 2014; Tornick et al. 2015) and in the field (Lyon 2003; Hunt et al. 2008; Low et al. 2009) supports an ability to distinguish among larger quantities in some animals. The ability to distinguish between two quantities has numerous advantages, as being able to accurately assess the difference in quantity between two options would be favored by natural selection in a number of situations. For example, the choice could involve food patches varying in size across the environment (Garland et al. 2012; Bogale et al. 2014), shifting mating tactic based on local sex ratio (Carazo et al. 2012; Řežucha and Reichard 2014), or choosing between groups of different sizes based on predation risk (Hager and Helfman 1991; Gomez-La Plaza and Gerlai 2011a). In fact, selection for numerical sense in any of the previously mentioned situations can be seen as an extension of Krebs' (1978) theory of optimal foraging, which states that when faced with two or more resource options, animals should choose the one that provides greater fitness.

In order to demonstrate an animal has the capacity to discriminate between two quantities, that animal must be influenced to do so. However, this influence does not

need to be in the form of providing a larger food reward to animals that choose the larger quantity. For example, in experiments on quantity discrimination in New Zealand robins (*Petroica longipes*), robins were allowed to take one mealworm from either a large or small cache. Despite the fact that the reward for quantity discrimination was the same regardless of their choice, the robins consistently chose mealworms from the larger cache (Hunt et al 2008, Garland et al. 2012). Similar results were found in wild jungle crows choosing between two plates of dog food (Bogale et al. 2014). The crows were allowed to assess two plates of food of differing quantities placed immediately adjacent to each other and after choosing a plate, consume all of the dog food regardless of whether they chose the larger or smaller quantity. The results that these two species significantly favored larger quantities, persisted regardless of the fact that the birds were rewarded no matter their choice.

In this sense, this effect may reflect other advantages of taking food from large vs. small caches. For example, a wide variety of caching bird species have been shown to assess a food resource before deciding whether to discard or cache the investigated object (Eurasian bullfinch, *Pyrrhula pyrrhula*, Greig-Smith and Crocker 1986; chickadees, *Poecile atricapillus,* and nuthatches, *Sitta Canadensis*, Heinrich et al. 1997; Mexican jays, *Aphelocoma wollweberi,* Jablonski et al. 2015). Thus a larger cache may provide consumers with more food options.

Conversely, individuals from social species may be more influenced by the behavior of other individuals than individuals of more solitary species. Though the theory of optimal foraging predicts that animals will choose the food patch that provides greater fitness between two or more options (Krebs 1978), overall quantity is not the

only factor affecting fitness and higher nutrient patches may have unforeseen risks and benefits. Indeed, in socially foraging birds, individuals prefer to feed in locations where conspecifics are feeding (cliff swallows, *Petrochelidon pyrrhonota,* Brown 1986; sandwich terns, *Sterna sandvicensis* Gotmark 1990; red-winged blackbirds, *Agelaius phoeniceus,* Avery 1994). These results have been replicated in other vertebrates where individuals prefer to feed with other group members (stickelbacks, *Gasterosteus aculeatus*, Harcourt et al. 2009; sheep, *Ovis aries,* Michelena et al. 2010). Preference for feeding with other individuals does not negate any innate quantity discrimination abilities but, for social species, overall quantity may be a secondary factor when foraging compared with a primary influencing factor of sociality.

When deciding to choose between two quantities, an organism's success at choosing its better option is limited by the cognitive systems it has available to distinguish between them. Two distinct systems for quantity discrimination, the "object file system" (OFS) and "analog magnitude system" (AMS) have been proposed based on both human (Feigenson and Carey 2005; Xu et al. 2005; Agrillo et al. 2012) and animal studies (Fetterman 1993; Farnsworth and Smolinski 2006; Gomez-Laplaza and Gerlai 2011a; Beran 2012). In OSF, animals store information on a small number of objects within their working memories to make precise distinctions in future comparisons (Rugani et al. 2014). Because OSF uses working memory to store relevant information on each object, it tends to have an upper limit of 3-4 objects (e.g., Feigenson et al. 2004; Revkin et al. 2008; Uller 2008). In contrast, AMS works by approximation and provides a fast and easy way for individuals to compare between choices based on the ratio of the larger to smaller quantities arranged in two lines or

quantities. So far, there is no upper limit to AMS. Instead, in accordance with Weber's Law, the ratio of the two choices (smaller to larger) determines individual accuracy, with smaller ratios leading to more accurate choices relative to larger ratios (Emmerton et al. 1997; Gilmore et al. 2011; Garland et al. 2014; Tornick et al. 2015).

The majority of species studied to date exhibit these ratio effects, with accuracy declining as the ratio between choices approached 1 (humans and guppies-Agrillo et al. 2012; pigeons-Brannon et al. 2001; coyotes-Baker et al. 2011; jungle crows-Bogale et al. 2011; chimpanzees-Beran 2012). The switch from OSF to AMS is thought to occur when the number of objects being compared exceeds 3-4 (Hunt et al. 2008; Armstrong et al. 2012). However, recent research on New Zealand robins found an exception to the ratio rule (Garland et al. 2012). New Zealand robins did not display any dependence on ratio when choosing the large group of mealworms until a much higher number of objects threshold was reached (up to 16 total objects) (Garland et al. 2012; Garland et al. 2014). The authors proposed that the OSF of New Zealand robins may have an extended number range (up to 16) and that this extension of OSF may be due to their reliance on extensive food-caching (Hunt et al. 2008; Garland et al. 2012). Indeed, life history of the species being studied undoubtedly plays a very important role in the evolved sophistication of their numerical sense and how they deploy it.

While species that rely on caching may inherently have a more sophisticated numerical sense, sociality is another life history factor that may also affect numerical sense. For group living species, keeping track of overall group size and also the relationships between group members and the size of changing coalitions within the group may affect individual fitness (Wittig et al. 2013; Hobson and DeDeo 2015; Platt et

al. 2016). In other cognitive tests, recent work on corvids demonstrates that the more social species outperformed less social species in serial reversal learning (social cache-reliant Pinyon jays, *Gymnorhinus cyanocephalus,* vs. territorial cache-reliant Clark's nutcrackers, *Nucifraga columbiana,* Bond et al. 2007), arbitrary non-spatial tasks (Pinyon jays and social somewhat cache-reliant Mexican jays vs Clark's nutcrackers and territorial somewhat cache-reliant Western scrub jays, *Aphelocoma californica*, Olson et al. 1995), and observational spatial memory tasks (Mexican jays vs Clark's nutcrackers, Bednekoff and Balda 1996a), regardless of the cache-reliance of the species. However, social factors have largely been controlled for in tests of quantity discrimination. Cronin (2014) conducted the only test of quantity discrimination that allowed multiple individuals to interact by observing the effect of ant colony size on number of scouts needed to reach a quorum for a new nest site. He found that in the Japanese ant (*Myrmecina nipponica*), the number of scouts needed to reach a quorum increased with colony size in a ratio dependent manner (Cronin 2014). He concluded that the ants employ AMS to quantify the number of nest mates, both within the colony and within the quorum (Cronin 2014). However, no study has investigated the role that sociality may play in influencing individual quantity discrimination ability and utilization.

I set out to determine how sociality affected individual quantity discrimination by comparing the highly social Mexican jay (MJ) with the closely related but territorial Western scrub jay (WJ). Both MJ and WJ are omnivorous, caching species. WJ are known to have episodic-like memories (Clayton and Dickinson 1998; Raby et al. 2007) and also for their ability to discern when another bird is watching them cache, leading them to re-cache later when given the opportunity (Dally et al. 2006). Indeed, WJ

flexibly adapt both caching and recovery strategies to reducing future pilfering when observed caching by conspecifics compared with caching alone (Emery et al. 2004). However, MJ may perform better in certain cognitive tasks than WJ, as WJ did not perform as well as the more social pinyon jays when performing serial reversal learning (Bond et al. 2007), and were also worse at arbitrary non-spatial tasks than the social MJ (Olson et al. 1995).

To test the role sociality plays in the quantity discrimination performance of wild MJ and WJ, I presented both species with two separate lines of peanuts to choose from with a range of ratios and number comparisons (Table 1) and determined their tendency to pick the larger quantity. Specifically, I will determine if sociality and the choices of other individuals influenced quantity discrimination in the two species. As quantity discrimination had not previously been studied in either of these species, I examined the roles ratio, total peanuts, difference between the lines, and social factors may play in quantity discrimination performance.

Methods

Study species

Two species participated in these trials, the cooperatively breeding, group living Mexican jay (*Aphelocoma wollweberi*, MJ) and the territorial, pair breeding Western scrub jay (*A. californica*, WJ). Both MJ and WJ were wild and free living and none of the birds were handled during the experiment. I observed 57 individual MJ from six flocks (7-14 members per flock) and 9 WJ from two different feeding stations participated in the two experiments. While the MJ and WJ groups are part of an ongoing comparative

study on the hormonal mechanisms of cooperative breeding (involving catching and collecting plasma from MJ and WJ of different breeding status), none of the birds had any prior cognitive experimental history. During the trials, each bird was identified based on its unique leg band combination.

Study site

This experiment was conducted between 4/12/14-4/28/14 in the foothills of the Chiricahua Mountains near the Southwestern Research Station in Portal, AZ (31° 53'N,109° 12'W). The MJ inhabit woodland dominated by oak-juniper-pine while the WJ live in mesquite dominated scrub. Prior to the start of the trials, all of the birds had been trained to come and eat peanuts at a designated feed site (one site each per MJ flock territory and two sites total for the WJ) when they heard a particular whistle. As both MJ and WJ are caching species (Vander Wall and Balda 1981) and accustomed to regular peanut treats at these sites, they respond quickly to exploit the peanuts before another animal preempts them. Each feed site was used in all 8 trials for the experiment.

Experimental design

Each trial had two separate lines of unequal numbers of shelled peanuts. To test if MJ and WJ exhibited quantity discrimination, peanuts were laid out in a straight line, 1 inch apart (Figure 18), while the two lines were 3 feet apart. I did not control for line length as this would have introduced lines with low and high peanut density and may have decreased the birds' ability to distinguish separate peanuts. The starting ratios ranged between 0.25 and 0.875 and the overall differences ranged between 1 (7 vs 8)

and 12 (4 vs 16) (Table 11). Trial order and placement of the larger line were randomized for each trial/feed site. To control for possible side bias, each flock/feed site had four trial starts with the larger quantity on the left and 4 trial starts with the quantity on the right. While the placement of the larger line was randomized, the spot on the ground where each line when (A and B) was always the same for each site/trials. Only one trial was run per day at each territory to control for possible effects of satiation between trials and to keep trial time consistent for each flock.

MJ are highly social birds that always came to feeding sites in groups. Even at both territorial WJ sites, multiple birds visited and participated during the experiments. When a bird landed and was making a choice between the sides, I recorded whether it was the first bird to choose, whether another bird was on the ground at the same time (and at which side) and any social interactions between the birds, as well as what side the focal bird ultimately chose. Post trial, these social effects (including what side the previous bird had chosen) were scored and analyzed along with line choice, ratio, and overall line difference to determine what factors most impacted an individual's choice.

Statistical analyses

The decision to allow the birds to remove all of the peanuts from both sides also created a number of new ratios, overall line differences, and total peanut numbers for each trial. For example, in one trial that began as 4 vs. 16, the subsequent seven removals created lines of:

4 vs 15, 3 vs 15, 3 vs 14, 3 vs 13, 3 vs 12, 2 vs 12, 1 vs 12

depending on the previous bird's choice, before ending when one of the lines was

completely removed. I included these new ratios and differences in my analysis but sample sizes for these unanticipated comparisons varied dramatically, with 5 vs 9 only appearing once in the MJ trials, while 1 vs 2 appeared 18 times in the same set of trials. To compare between line differences with similar sample sizes, I grouped the line difference analyses as low (1-4), medium (5-8), and high (>8). Similarly, for the ratio analyses, I grouped the ratios as 0.01-0.125, 0.126-0.25, 0.26-0.375, 0.376-0.5, 0.51-0.625, 0.626-0.75, 0.76-0.875, and 0.876-1.

To determine whether each bird species showed an overall preference for larger quantities, I performed a Chi-square analysis on whether each species was more likely to pick the larger line across all choices/trials. I also used a Chi-square analysis to analyze whether MJ and WJ were more or less likely to pick the larger quantity when differences between the two lines was Low (1-4), Medium (5-8), or High (>8). I ran generalized linear mixed models (GLM) in R version 2.15.3 (R core team, 2013) to determine whether sociality, ratio, and/or line difference was affecting each bird's choice of line. Model selection was based on AIC values. For each comparison, I considered subjects' choice as ''successful/accurate'' when they chose peanuts from the line with a greater number of peanuts. Specifically, I tested the following factors: ratio, Line Difference, Total Peanut quantity, another bird in the arena, and following the previous bird's choice (Follow). Trial order, flock ID, and bird ID were treated as random effects. When Trial order was treated as a fixed effect, it did not significantly affect line choice (MJ, P=0.382; WJ, P=0.461) (Table 12). I calculated the ratio of the peanut lines by dividing the smaller line number into the larger line number (i.e. 4/8 = 0.5). Total Peanut was determined by combining the total number of each line (i.e. 4 vs 6 = 10).

Results

Overall, WJ, but not MJ, were significantly more likely to pick the larger line across all choices/trials (WJ: 61.57%, χ^2 (1, N=242)=12.96, P=0.0003; MJ: 51.87 %, χ^2 (1, N=613)=0.863, P=0.353) (Figure 19A). However neither MJ nor WJ picked the larger line more than chance when considering the choices of the first bird only (WJ: 55.55%, χ^2 (1, N=18)=0.222, P= 0.637; MJ: 51.06 %, χ^2 (1, N=47)=0.021, P=0.884) (Figure 19A). When Line Difference was subdivided into Low (1-4), Medium (5-8), and High (>8), MJ were significantly more likely to choose the larger quantity in the low category only (MJ: Low, 56.1%, χ^2 (1, N=344)=5.12, P=0.02; Med, 49.3%, χ^2 (1, N=154)=0.026, P=0.87; High, 42.6%, χ^2 (1, N=115)=2.513, P=0.11), whereas WJ choices remained significant across all categories, indicating that WJ continued to select peanuts from the larger line (WJ: Low, 58.78%, χ^2 (1, N=148)=4.56, P=0.03; Med, 62.8%, χ^2 (1, N=70)=4.62, P=0.03; High, 75.0%, χ^2 (1, N=24)=6, P=0.01) (Figure 19B).

For the GLMs, there was a significant interaction effect between Ratio and Line Difference for WJ (P=0.048) but not MJ (P=0.508) tendency to pick the larger line (Figure 20AB) (Table 12). As ratios approached 1, MJ were less likely to choose the line with more peanuts irrespective of the line difference (although this was not significant), whereas WJ were less likely to choose the line with more peanuts only when the line differences were Medium and High (Figure 20AB). For WJ, Line Difference was a significant factor (P=0.038), with the percentage of birds that chose the larger line increasing as the difference between the larger and smaller pile increased (Figure 19B). In contrast, for WJ, Ratio was only a significant factor through its interaction with line difference (Ratio alone: P= 0.598, Ratio by LD: P=0.048) (Figure 20B, 21). When Line

Difference was Low, the percentage of WJ that chose the larger line was not affected by Ratio. However, when Line Difference was Med or High, the percentage of birds that chose the larger line declined (Figure 20B). For the MJ, the percentage of birds that chose the larger line decreased as both the Ratio and the Line Difference between the larger and smaller pile increased (Figure 20A, 21); however, neither Ratio nor Line Difference were significant factors in the GLM model optimized according to the lowest AIC score (P=0.508; Table 12).

Unlike WJ, the factors most significant for MJ involved a strong social component related to whether birds choose the same line as the previous birds (followers) or whether they choose the other line. For MJ, there were significant interactions between Total Peanut, Line Difference, and Follow (P=0.0007) and Line Difference and Follow (P=0.005) (Figure 22A). As Line Difference grew, almost 100% of the MJ that did not follow the previous bird selected the larger pile, compared to 40% of MJ that did follow the previous bird (Figure 22A). In contrast, WJ were more likely to choose the larger pile as Line Difference increased, with no differences between birds that followed the previous bird vs. those that did not follow (P=0.847) (Figure 22B). The other social factor tested, the presence/absence of another bird on ground, was not significant either for MJ (P=0.069, N=137) or for WJ (P=0.529, N=42).

Discussion

I set out to establish the quantity discrimination abilities of MJ and WJ, to determine what factors most influenced their success, and to understand the role sociality may play on their quantity discrimination abilities. This is the first study to

examine the quantity discrimination abilities of either species and did so in a natural setting that allowed for interactions between conspecifics. However, these social interactions may obscure the discrimination abilities of each species in the absence of social cues. Therefore, these results do not establish the basal quantity discrimination abilities for either species in a controlled environment (i.e. a lab setting). Instead, this study provides evidence for how each species utilizes inherent quantity discrimination abilities when also processing additional external factors to make foraging decisions in nature. WJ discriminated between the two lines and chose the larger line more often than by chance overall and for all line difference categories (Low, Med, High). Conversely, MJ did not choose the larger line when comparing line differences overall and only displayed a significant preference for the larger quantity when line differences were Low. This result is likely due to the interaction effect found in MJ between line difference and following the previous bird's choice. MJ that followed the previous bird were significantly less likely to choose the larger line when the total line difference was categorized as High but not for Low or Med.

These results reflect the fact that for performance in quantity discrimination experiments, each individual's choice encompasses both ability and internal and external influences. While both MJ and WJ would only remove one peanut at a time, then fly off and cache the peanut before returning to make another choice, my results suggest that WJ were both influenced by and able to select peanuts from the larger quantity line. Conversely, MJ seemed only influenced by and able to discriminate between two quantities when their difference was low (Figure 2). However, due to grouping line difference in discrete Low, Med, and High treatments (to minimize

differences in sample sizes) as opposed to treating it as a continuous variable, the overall affects of line difference on MJ choice may have been weakened.

The key difference between the species may lie in what influences individual choice in situations with varying quantities. For WJ, the interaction between ratio and line difference significantly affected success, but while line difference was a significant factor on its own, ratio was not. These results suggest that, like the New Zealand robin, WJ may have an extended OSF number range due to their extensive caching (Hunt et al. 2008; Garland et al. 2012) and may make the switch to relying on ratio and using AMS much later than 3-4 objects. Similarly to WJ, for MJ ratio did not affect choice, although other non-social factors such as line difference and total peanuts did affect their success in picking the larger line (Table 2). For MJ, the factors that most significantly affected choice were social, although they were significantly influenced to pick the larger quantity when the difference between the lines was low (Figure 2B).

While my results suggest that when WJ and MJs discriminate between two quantities they employ an extended OSF, a recent study on a related corvid, Clark's nutcracker, found that ratio was an important determinant (according to Weber's Law), suggesting reliance on AMS (Tornick et al. 2015). Interestingly, all three species of corvids rely on food caches but while Clark's nutcrackers are highly dependent on cache recovery, both MJ and WJ are generalist omnivores that rely less heavily on caching. One potential explanation for the differences in cognitive systems employed in quantity discrimination between MJ and WJ is that it is determined by tendency of each species to pilfer the caches of conspecifics. MJ and WJ (and New Zealand robins, which also rely on extended OSF) experience high amounts of cache pilferage by

conspecifics (robins, Menzies and Burns 2008; WJ, Dally et al. 2006; MJ, Bednekoff and Balda 1996a). Indeed, WJ have become well know for their ability to determine when they are being observed while caching and to take measures to reduce conspecific cache theft (including caching at a distance, in the shade, behind objects, and re-caching when the conspecific leaves) (see Clayton et al. 2007). Consequently, this increased pressure from conspecific cache theft may drive increased OSF; individuals need to keep track not only of their own caches, but also which of the many caches around them are worth pilfering.

The effect of sociality on quantity discrimination was the most important distinction between MJ and WJ. While quantity difference mattered in some scenarios for both species, the choices other birds made influenced only MJ accuracy. Not surprisingly, the highly social MJ were greatly influenced by what the other individuals in their flock were doing and this was reflected in their choice of peanut lines. However, following the other birds led MJ to focus more on the smaller lines. This was especially true as the difference between the two lines grew; with birds that followed the previous bird much more likely to pick the smaller line at high line difference (Figure 3). As observing conspecifics provides important information to individuals, such as predatory risk (Handegard et al. 2012) and resource quality/abundance (Krause et al. 2010), subsequent MJ may choose to follow the first few birds' choices to a particular line due to lower perceived risk or perceived higher rewarding payoff.

These results support general findings on social foraging in birds, where flocking birds prefer to feed in locations where conspecifics are feeding (Brown 1986, Gotmark 1990, Avery 1994). Indeed, social factors can be such a potent factor that Burmese fowl

(*Gallus gallus*) prefer to feed where another bird was seen feeding up to 48 hours after the initial observation (McQuoid and Galef 1992; 1993). This may also explain the interaction effect between Line Difference and Follow in MJ, where MJ that did not follow the previous bird became more accurate in choosing the larger quantity as difference between the two lines increased while birds that chose to follow the previous bird become much less accurate.

Another social factor that may have led to MJ following other birds to the smaller lines is dominance within the flock. Though I did not determine dominance for the flocks as a part of this study, dominance hierarchies are well established for MJ flocks. Males tending to be dominant to females and the oldest males tending to have the highest status, while juvenile yearlings also experience relatively high status (Barkan et al 1986). During the trials, some individual MJ would wait in the trial area for other birds to make their peanut choice (usually based on who had arrived first) before selecting a peanut from the line the previous bird had selected (*personal observation*). While I never observed any fights among MJ during any of the trials, it is possible that this waiting and selecting the same line as the previous bird was influenced by the dominance hierarchies within the flocks, with more subordinate birds waiting until more dominant birds were finished.

While this strong social effect on foraging did negatively affect Mexican jay quantity discrimination, it seems to accurately illustrate how social individuals employ quantity discrimination while foraging in natural settings. Future studies should compare other closely related species that differ in sociality to determine if sociality plays a consistent role in quantity discrimination across species.

Conclusions

Overall, this study demonstrates the important role of sociality in performance on a quantity discrimination task, a previously overlooked factor. Because these trials were run in the wild on untrained birds, the results suggest the factors most important when individuals are choosing between patches of different quantities can vary widely between even closely related species. For the highly social MJ, the choices of other birds were much more important than whether or not an individual picked a peanut from the larger line, while the territorial WJ seemed to ignore the choices of the other individuals and focus more on total peanut amount in each line. While the majority of studies on quantity discrimination have established this ability across a number of species, because they took place largely in highly artificial lab environments, we have yet to determine how species utilize quantity discrimination when making choices in a natural setting. To understand how and why quantity discrimination evolves, we must determine the other factors that influence quantity choices and not just determine whether animals can determine large or small quantities. My results demonstrate that sociality is important and may strongly affect individual choice in quantity discrimination experiments.

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Tables

Line A	Line B	Ratio	Line Difference	Total Peanut
2	8	0.25	6	10
4	16	0.25	12	20
4	8	0.5	4	12
8	16	0.5	8	24
6	8	0.75	2	14
12	16	0.75	4	28
7	8	0.875	1	15
14	16	0.875	2	30

Table 11. The starting number comparisons, ratios, line differences and total peanuts displayed for each of the eight trials investigated across the experiment.

Species	Model	Random Effects per model			Model Subset	Estimate ± SE	AIC Score	Z value	P value
		Category	*Variance*	*SD*					
MJ	Bird on ground	BirdID	0.055	0.235	Bird on ground	0.352±0.194	847.4	1.815	0.069
		Trial	0.133	0.364					
		FlockID	0.022	0.149					
	Ratio by LD	BirdID	0.084	0.29	Ratio by LD	-0.216±0.123	835.4	-1.76	0.078
		Trial	0.072	0.268	Ratio	-1.236±0.636		-1.94	0.052
		FlockID	0.01	0.099	LD	-0.095±0.049		-1.9	0.058
	TP by Follow by LD	BirdID	0.04	0.2	TP by Follow by LD	-0.039±0.012	**816.5**	-3.38	**7.00E-04**
		Trial	0.039	0.197	Follow by LD	0.521±0.189		2.762	**0.006**
		FlockID	0.018	0.135	TP by LD	0.006±0.007		0.855	0.393
					TP by Follow	0.079±0.044		1.783	0.075
					TP	-0.046±0.030		-1.51	0.132
					Follow	-1.238±0.692		-1.79	0.073
					LD	-0.092±0.125		-0.73	0.463
	Trial Order	BirdID	0.051	0.226	Trial Order	0.025±0.037	853.6	0.692	0.489
		FlockID	0.037	0.193					
WJ	Bird on ground	BirdID	0.121	0.348	Bird on ground	0.239±0.38	326.9	0.63	0.529
		Trial	0.175	0.418					
		FlockID	4.29E-10	2.10E-05					
	Ratio by LD	BirdID	0.156	0.395	Ratio by LD	-0.640 ± 0.325	**321**	-1.97	**0.048**
		Trial	0.215	0.463	Ratio	0.674±1.279		0.527	0.598
		FlockID	1.27E-10	1.13E-05	LD	0.319±0.153		2.075	**0.038**
	TP by Follow by LD	Trial	0.358	0.599	TP by Follow by LD	-0.033±0.020	324.8	-1.67	0.096
		BirdID	0.166	0.408	Follow by LD	0.607±0.338		1.795	0.073
		FlockID	8.01E-08	2.00E-04	TP by LD	-0.003 ± 0.013		-0.26	0.793
					TP by Follow	0.049±0.078		0.626	0.531
					TP	0.012±0.052		0.231	0.818
					Follow	-0.987±1.121		-0.88	0.379
					LD	0.170±0.231		0.735	0.462
	Trial Order	BirdID	8.15E-02	2.86E-01	Trial Order	0.043±0.058	327.7	0.738	0.461
		FlockID	3.74E-10	1.94E-05					

Table 12. Generalized linear model scores for the models and factors used to determine the factors affecting line choice. Model selection was based on AIC values.

Figures

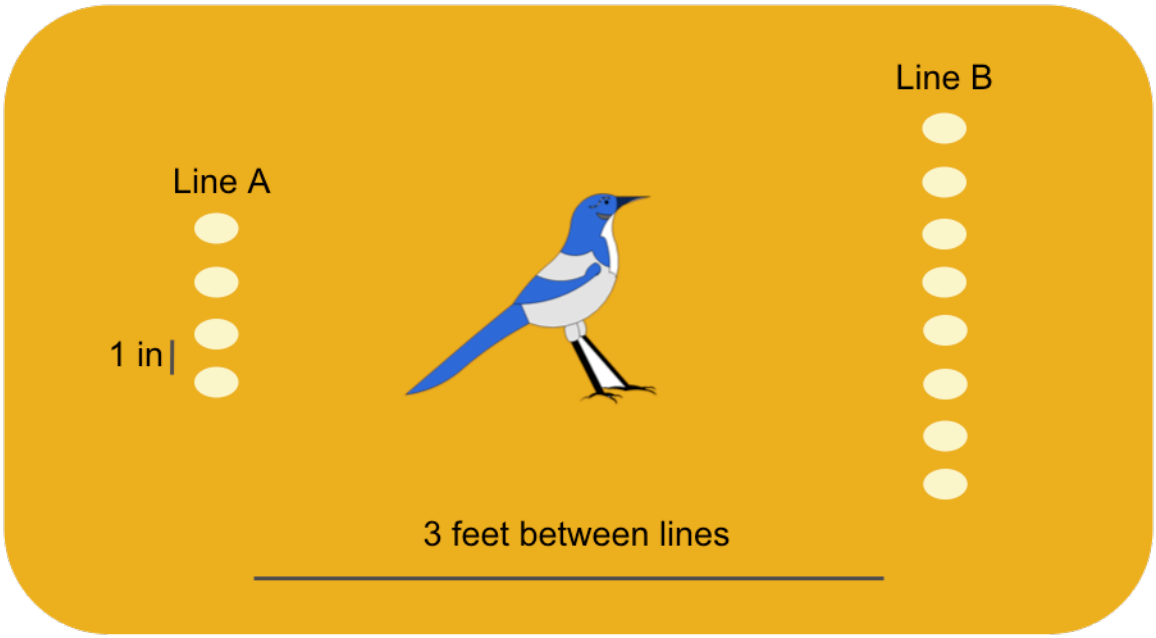

Figure 18. *Experimental design*: For the start of each trial, peanuts were laid out in straight lines, 1in apart in two separate lines. The two lines were three feet apart and the same line sites were used for every trial.

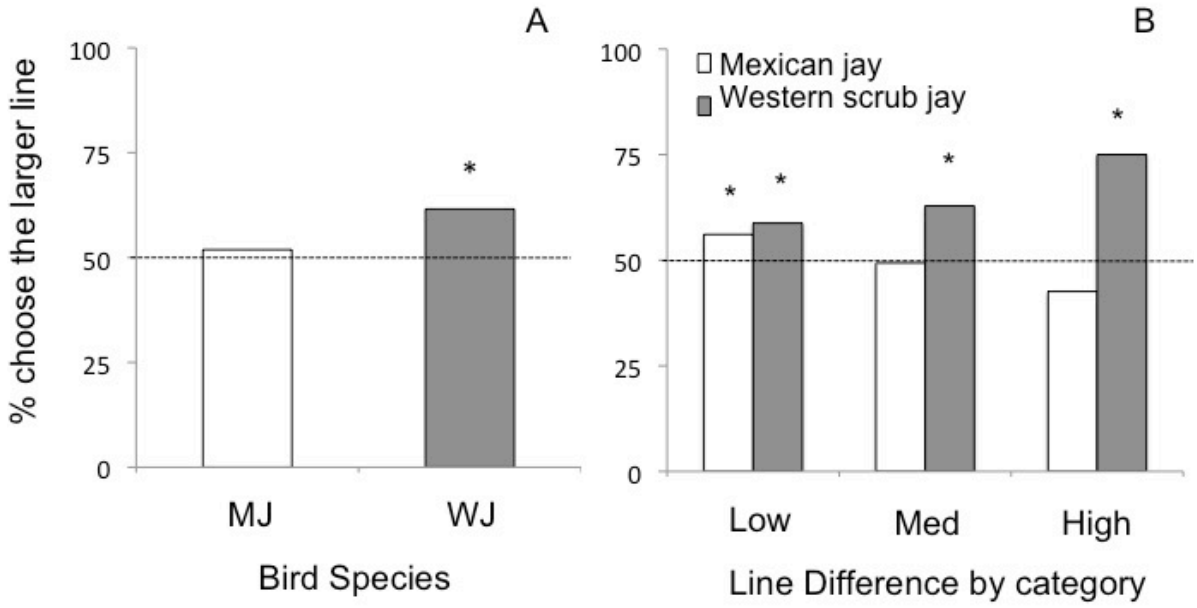

Figure 19AB. The percentage of birds that chose for the larger line as a function of Line Difference. Low (<5 peanut difference), Med (5-8), High (>8). When percentages are significantly different from 50%, the bars are marked with "∗"(Chi-square, P<0.05).

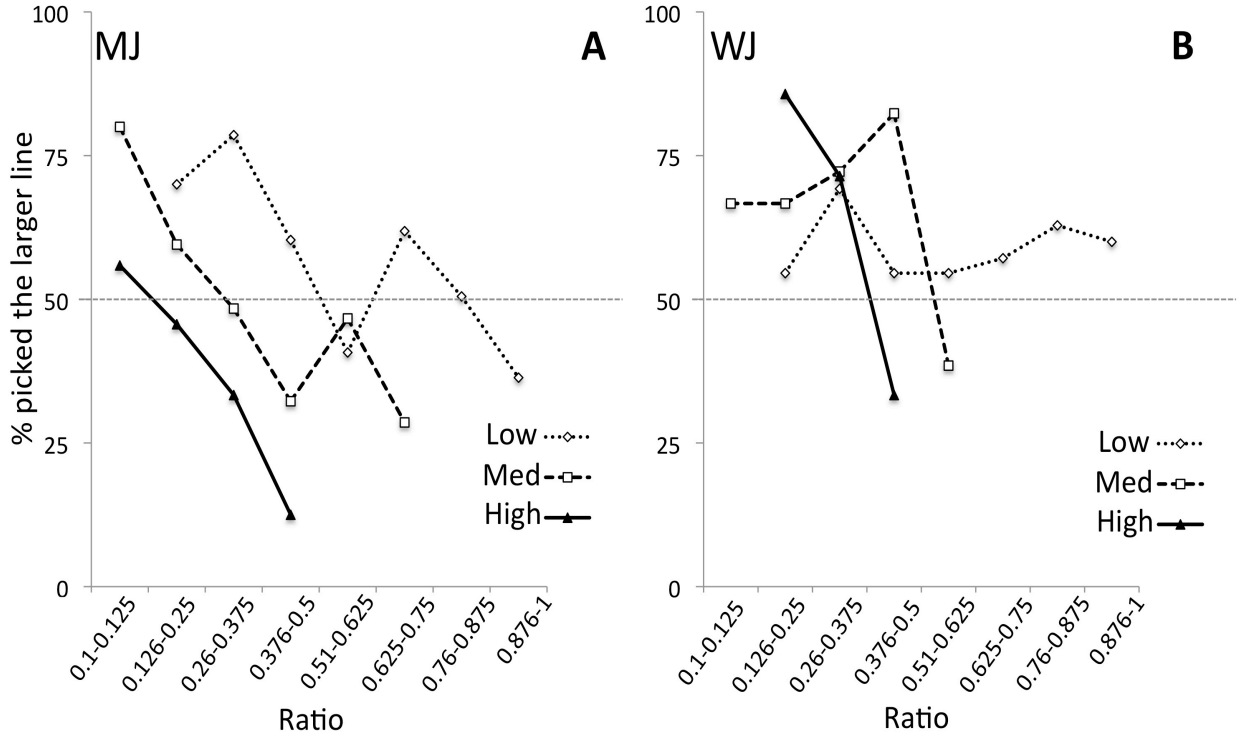

Figure 20 AB. The percentage of birds that chose the larger line as a function of the interaction between the ratio and line difference of the larger and smaller line of peanuts. The interaction is significant only for WJ (P-values correspond to the interaction term in GLM).

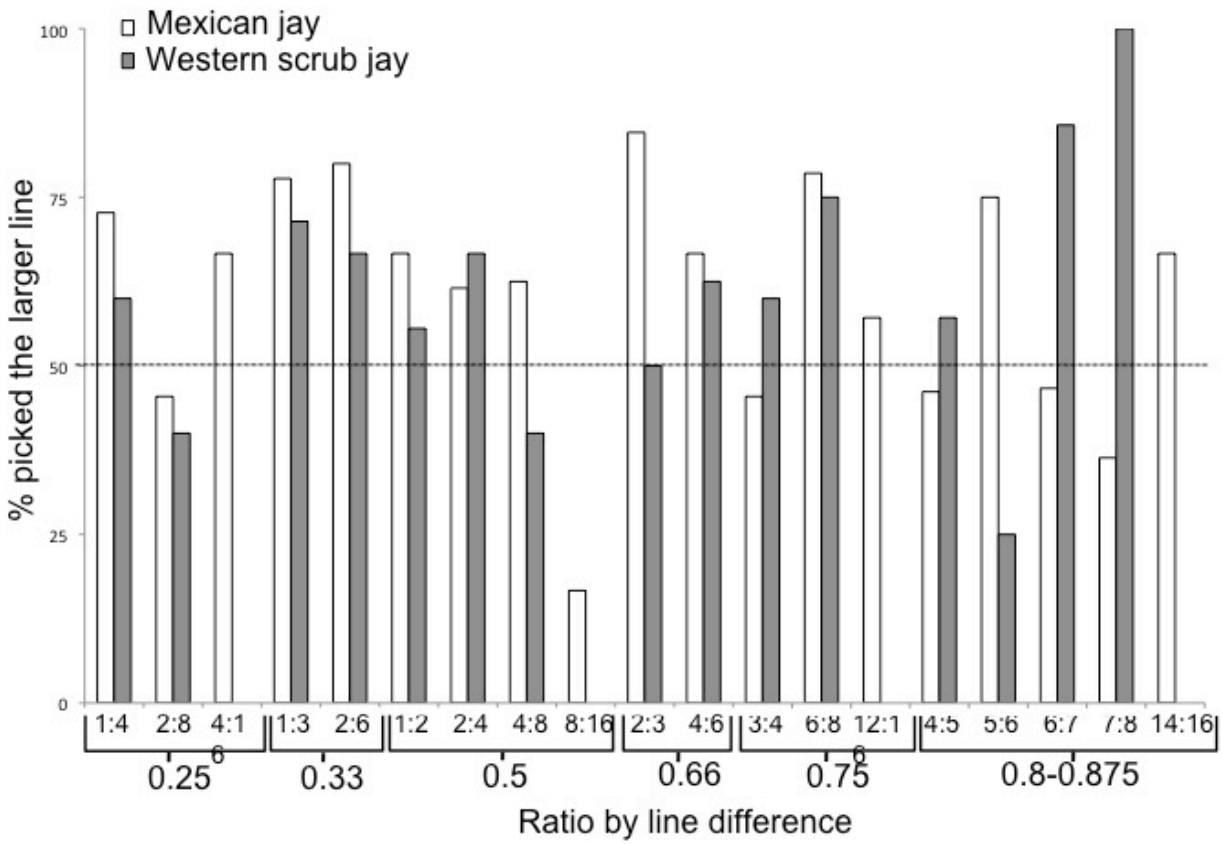

Figure 21. The percentage of birds that chose the larger line as a function of the ratio between the larger and smaller line of peanuts. Ratio does not play a significant role for either Mexican jays' (P=0.508) or Western scrub jays' (P= 0.5981) accuracy, according to GLM. Accuracy did not decline as ratios approached 1 and did not support the predictions of Weber's law.

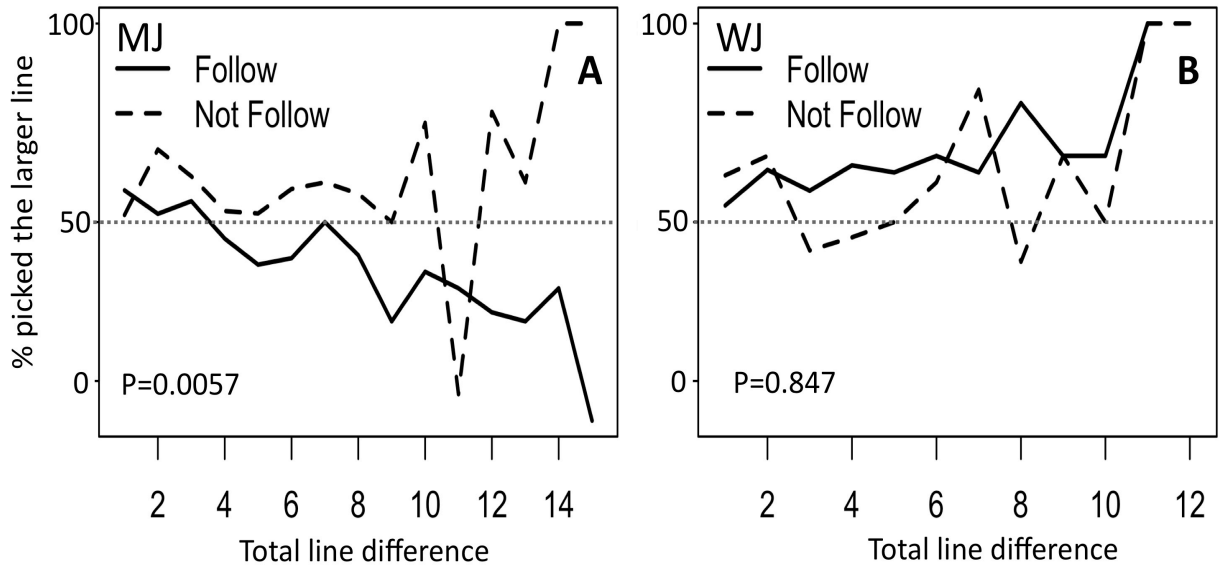

Figure 22AB. The percentage of birds that chose the larger line as a function of the interaction between the Line Difference of the larger and smaller line and whether the bird went to the same line the previous bird had chosen (Follow) or to the opposite line (Not Follow). MJ that followed the previous bird were less likely to choose the larger line when the total line difference was large (P values refer to the interaction effect according to GLM).